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Bioengineering of β-galactosidases for their use in the food industry

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Contents

Abbreviations

3GalGlu	**3**-**Gal**actosyl-**Glu**cose
4GalLac	**4**-**Gal**actosyl-**Lac**tose
6GalGal	**6**-**Gal**actosyl-**Gal**actcose (galactobiose)
6GalGlu	**6**-**Gal**actosyl-**Glu**cose (allolactose)
6GalLac	**6**-**Gal**actosyl-**Lac**tose
µM	**microM**olar
µm	**microm**eter
ºC	**C**elsius degree
Å	Armstrong
β-PNPG	**P**-**N**itro**P**henyl-**β**-D-**G**alactopyranoside
AnβGal	***A****spergillus* ***n****iger* **β**-**Gal**actosidase
AoβGal	***A****spergillus* ***o****ryzae* **β**-**Gal**actosidase
CASTing	**C**ombinatorial **A**ctive **S**ite **T**est
Da	**Da**lton
dhA	**d**i**h**ydro**A**lanine
DNA	**D**eoxyribo**N**ucleic **A**cic
DSF	**D**ifferential **S**cnning **F**luorimetry
DTNB	5, 5´-**D**i**T**hiobis (2-**N**itro**B**enzoic acid)
EndoH	**Endo**glycosidase **H**
FDA	**F**ederal **D**rug **A**dministration
g	**g**ram
GH35	**G**lycoside **H**ydrolase family **35**
GlcNAc	**N**-**Ac**etyl**Gl**u**c**osamine
GOS	**G**alacto-**O**ligo**S**accharides
GRAS	**G**enerally **R**ecognized **A**s **S**afe
h	**h**ours

HMO	**H**uman **M**ilk **O**ligosaccharides
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
IPTG	**I**so**P**ropyl **T**hio-β-D-**G**alactoside
K	**K**elvin degree
Kcat	turnover number
kDa	**k**ilo**Da**lton
KlβGal	***K****luyveromyces* ***l****actis* **β**-**Gal**actosidase
Km	Michaelis constant
L	**L**itre
LC	**L**iquid **C**hromatography
MALDI	**M**atrix-**A**ssisted **L**aser **D**esorption/**I**onization
min	**min**ute
mL	**m**ili**L**iter
mM	**m**ili**M**olar
mmol	**m**ili**mol**
MS	**M**ass **S**pectrometry
PAGE	**P**oly**A**crylamide **G**el **E**lectrophoresis
PCR	**P**olymerase **C**hain **R**eaction
PDB	**P**rotein **D**ata **B**ank
PEG3350	**P**oly**E**thylene **G**lycol **3350**
PETG	**P**henyl**E**thyl **T**hio-β-D-**G**alactoside
pH	**H**ydrogen **p**otential
PsβGal	***P****enicillium* **s**p. **β**-**Gal**actosidase
RMS	**R**oot **M**ean **S**quare
rpm	**r**evolution **p**er **m**inute
SCFA	**S**hort **C**hain **F**at **A**cids

SDS	**S**odium **D**odecyl **S**ulphate
SEM	**S**canning Electron **M**icroscopy
TBS	**T**ris-**B**uffered **S**aline
Tm	**m**elting **T**emperature
TrβGal	***Tr****ichoderma reesei* **β**-**Gal**actosidase
U	enzymatic **U**nits
Vmax	**max**imal **V**elocity
WT	**W**ild **T**ype

Short Abstracts

ABSTRACT

β-galactosidases are enzymes required in a large number of applications by food industry. In this thesis we engineered the β-galactosidases from *Aspergillus niger* and *Kluyveromyces lactis* in order to obtain more suitable forms for industrial uses. *Aspergillus niger* β-galactosidase was expressed in yeast, purified by affinity chromatography and crystallized to perform X-ray diffraction experiments. The three-dimensional structures of the enzyme in its native form and in complexes with five oligosaccharides were solved. The results gave insights into specificity determinants of GH35 β-galactosidases catalysis and this knowledge allowed engineering of the enzyme to achieve new variants with increased transgalactosylation activity. Moreover, thermostable mutants of β-galactosidase from *Kluyveromyces lactis* were obtained by performing a rational design strategy. The introduction of new disulfide bonds among subunit interfaces also produced a noticeable rise in the catalytic efficiency of the mutants. This effect was attributable to quaternary structure stabilization, which increases the proportion of the highest active oligomeric forms in the equilibrium. Finally, immobilization studies using two different supports showed that in the mutant the loss of catalytic activity after immobilization, even after several reutilization cycles, was lower than in the native protein. Therefore, this mutant variant performed better than the native form during immobilization, which represents a new advantage for industrial applications.

RESUMEN

Las β-galactosidasas son enzimas utilizadas en numerosas aplicaciones de la industria alimentaria. En esta tesis se modifican las β-galactosidasas de *Aspergillus niger* y de *Kluyveromyces lactis* para obtener variantes adecuadas para su uso industrial. La β-galactosidasa de *Aspergillus niger* se expresó, purificó y cristalizó para llevar a cabo experimentos de difracción con rayos X. Se resolvieron las estructuras tridimensionales de la enzima en sus formas nativa y acomplejada con cinco oligosacáridos. Los resultados ayudan a entender las claves que determinan la especificidad en la catálisis de las β-galactosidasas de la familia GH35 y permiten la modificación de la enzima para obtener nuevas variantes con mayor actividad de transgalactosilación. Además, se obtuvieron mutantes de β-galactosidasa de *Kluyveromyces lactis* mediante una estrategia de diseño racional. La introducción de nuevos puentes disulfuro en la zona de interacción de las subunidades también produjo una importante subida en la eficiencia catalítica de los mutantes. Finalmente, los estudios de inmovilización con dos soportes diferentes mostraron que en el mutante la pérdida de actividad catalítica después de la inmovilización fue inferior que en la proteína nativa. Por lo tanto, esta variante mutante se comportó mejor que la forma nativa durante la inmovilización, lo que implica una nueva ventaja para su uso en aplicaciones industriales.

RESUMO

As β-galactosidasas son enzimas utilizadas en numerosas aplicacións da industria alimentaria. Nesta tese modifícanse as β-galactosidasas de *Aspergillus niger* e de *Kluyveromyces lactis* para obter variantes axeitadas para o seu uso industrial. A β-galactosidasa de *Aspergillus niger* expresouse, purificose e cristalizouse para levar a cabo experimentos de difración con raios X. Resolvéronse as estruturas tridimensionais da enzima nas súas formas nativa e acomplexada con cinco oligosacáridos. Os resultados axudan a entender as claves que determinan a especificidade na catálisis das β-galactosidasas da familia GH35 e permiten a modificación da enzima para obter novas variantes con maior actividade de transgalactosilación. Ademais, obtivéronse mutantes de β-galactosidasa de *Kluyveromyces lactis* mediante unha estratexia de deseño racional. A introdución de novas pontes disulfuro na zona de interacción das subunidades tamén produciu unha importante subida na eficiencia catalítica dos mutantes. Finalmente, os estudos de inmovilización con dous soportes diferentes mostraron que no mutante a perda de actividade catalítica despois da inmovilización foi inferior que na proteína nativa. Polo tanto, esta variante mutante comportouse mellor que a forma nativa durante a inmovilización, o que supón unha nova vantaxe para o seu uso en aplicacións industriais.

Introduction

1. β-GALACTOSIDASES

Enzymes or biocatalysts have been used in food processing for millennia. Archaeological studies register applications for bread baking, cheese making and beer brewing for more than 8000 years ago (Vasic-Racki, 2006).

Food market has an increased demand of healthy and high quality products and enzymes are essential tools in order to develop efficient industrial processes. Nowadays, global sales of enzymes for food industry are about 1.3 billion USD per year, standing out the milk and dairy market area, with about 401.8 million USD per year (Jemli et al., 2016).

Some of the most used enzymes in dairy industry are β-galactosidases or lactases. β-galactosidases catalyze the hydrolysis of β-galactosides into their monosaccharides, and the trans-galactosylation of other glycosyl acceptors, depending on initial reaction conditions (Husain, 2010; Oliveira et al., 2011).

1.1. Classification

β-galactosidases (β-D-galactohydrolase EC 3.2.1.23) belong to a big class of enzymes known as Glycosyl-Hydrolases (GH) that include trans-glycosidases and glycosidases. β-galactosidases are glycosidases which are responsible for hydrolysis and/or trans-glycosylation of glycosidic bonds (Cantarel et al., 2009). According to sequence similarities, GH class is divided in 135 families. Diverse β-galactosidases are found in families GH1, GH2, GH3, GH35, GH43, GH50 and GH59. All these families belong to the clan (or family group) GH-A.

Glycosidases can also be classified according to their catalytic mechanism, in *retaining glycosidases* and *inverting glycosidases*. Clan GH-A and, therefore, all β-galactosidases belong to *retaining glycosidase* group.

1.2. Catalytic reaction

The catalytic retaining mechanism characteristic of β-galactosidases and other glycosidases includes two main steps. In the first step or glycosylation, a residue that acts as general acid/base catalyst donates a proton to the glycosyl oxygen atom, and other ionizable amino acid acts as nucleophile by forming an enzyme-sequestered covalent-intermediate between its carboxyl and the C1 of sugar moiety (galactose in β-galactosidases). This step facilitates the release of the leaving group. In the second step of the reaction mechanism, the acceptor molecule is activated by the deprotonated acid/base catalytic residue that makes a nucleophilic attack on the covalent intermediate formed in the first step and releases the product (Vuong and Wilson, 2010) (Figure 1).

The final product of the reaction depends on the nature of the acceptor molecule in the second step of the process. When the acceptor molecule is water, disaccharides or oligosaccharides are hydrolyzed. On the other hand, oligosaccharides are formed when the glycosyl moiety is accepted by another saccharide (trans-galactosylation reaction in β-galactosidases) (Gosling et al., 2010; Sangwan et al., 2011) (Figure 1).

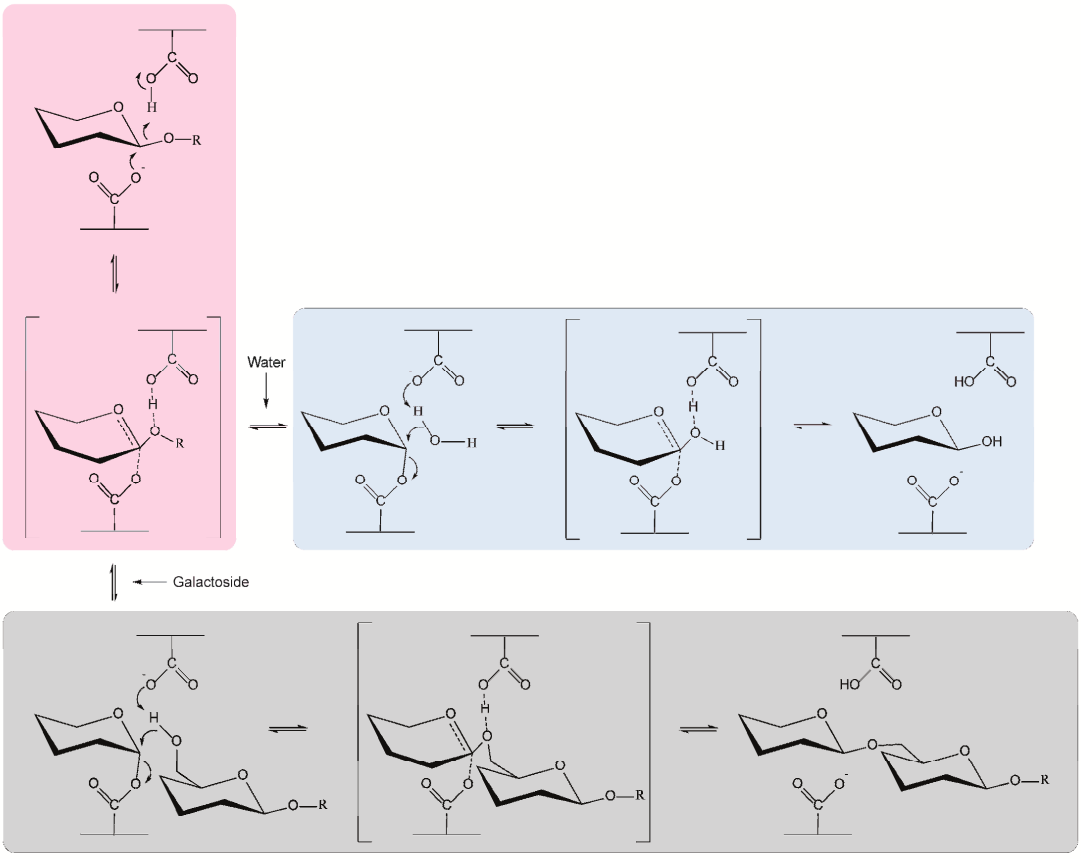

Figure 1: Catalytic retaining mechanism of β-galactosidases. Pink rectangle contains glycosylation step. Blue rectangle contains second step of the reaction when the acceptor molecule is water (hydrolysis) and grey rectangle contains second step of the reaction when the acceptor molecule is a galactoside (transgalactosylation).

The decisive factor that determines the reaction type (hydrolysis or transfer reaction) is the initial concentration of each of the potential glycosyl acceptors. In β-galactosidases, a high concentration of lactose will favor the transgalactosylation and production of galactooligosaccharides (Gosling et al., 2010). Unfortunately, lactose solubility is low (Machado et al., 2000). Therefore, applications that need transgalactosylation reactions must be performed at relatively high temperatures, in order to increase lactose solubility and allow high concentrations of this disaccharide in the

reaction mixture. This requirement limits the use of β-galactosidases with low melting temperature in these processes (Park and Oh, 2010).

1.3. *Aspergillus niger* β-galactosidase

β-galactosidase from the filamentous fungus *Aspergillus niger* (AnβGal) has been traditionally used on the dairy industry. Like other fungal enzymes, it has an acidic pH optimum (3.5-4.5), which explains its extended use in the hydrolysis of acid whey, a by-product of cheese factories (Panesar et al., 2006). The extracellular expression of AnβGal, in contrast with intracellular production of other β-galactosidase derived from yeasts, is one of its most interesting features because it allows important cost-saving in purification steps. Moreover, heterologous expression systems of AnβGal in hosts like *Pichia pastoris* or *Saccharomyces cerevisiae* were developed, which facilitate even more the industrial production of the enzyme (Dragosits et al., 2014; Oliveira et al., 2011). Furthermore, fungal β-galactosidases are characterized by a high temperature optimum and a reasonable protein stability, which allow them for being interesting options in applications that need high temperatures, such as synthesis of galactooligosaccharides (Panesar et al., 2006). Finally, the GRAS status of *A. niger* (Schuster et al., 2002) supports the use of its enzymes in the dairy industry.

AnβGal, like most of the eukaryotic β-galactosidases, is a member of family GH35. Although the tridimensional structure of AnβGal has been resolved during the course of this work, other fungal β-galactosidase structures from *Penicillium* sp. (PDB accession code 1TG7), *Trichoderma reesei* (PDB accession code 3OG2) and *Aspergillus oryzae* (PDB accession code 4IUG) are already available in the Protein Data Bank (PDB). The study of the three previous fungal structures (Maksimainen et al., 2011; Maksimainen

et al., 2013; Rojas et al., 2004) shows that, in all cases, the enzyme is arranged in six domains. Five of these domains (anti-parallel β-sandwich structures) form a horseshoe surrounding the catalytic domain ($(\alpha/\beta)_8$ barrel structure). The catalytic site is very similar in these structures, and all of them are monomeric (Figure 2). Sequence similarity between AnβGal and these three fungal β-galactosidases predicts similar folding and structure.

Figure 2: Structural alignment of GH35 fungal β-galactosidases in cartoon tube representation. β-galactosidases included are from *Penicillium* sp. (green color) *Trichoderma reesei* (red color) and *Aspergillus oryzae* (grey color).

While AnβGal has been mainly used in industrial applications that require a high hydrolytic activity, other fungal β-galactosidases like the one from *Aspergillus oryzae* (AoβGal) have been extensively utilized to synthesize transgalactosylation products (Gosling et al., 2010; Park and Oh, 2010). It

was observed that, although AnβGal has some transgalactosylation ability, its enzymatic activity tends to be hydrolytic, with better catalytic properties compared with other fungal enzymes like AoβGal (Dragosits et al., 2014).

Despite the presumably similarity, the study of its structure might explain the structural keys that define the catalytic preferences of GH35 fungal β-galactosidases, which would reveal interesting implications for the biotechnological directed-improvement of these β-galactosidases.

1.4. *Kluyveromyces lactis* β-galactosidase

The β-galactosidase from the yeast *Kluyveromyces lactis* (KlβGal) is one of the most used enzymes in food industry, especially for the production of lactose-free milk products. Many commercial preparations (Maxilact, Lactase or Lactozyme) used in food industry include KlβGal as their main component (Panesar et al., 2006).

KlβGal, like most yeast enzymes, is more active at neutral pH. This, in conjunction with its high hydrolytic activity, makes it suitable for hydrolysis of lactose in milk (Husain, 2010; Rubio-Texeira, 2006). Moreover, the recognition of *K. lactis* as GRAS (general recognized as safe), reinforces the convenience of this source of the enzyme for food industry, because the nonpathogenic and nontoxic nature of the microorganisms utilized for enzyme preparations defines their safety grade (Jemli et al., 2016).Besides, KlβGal has been used to obtain transgalactosylation products (Cardelle-Cobas et al., 2011; Chockchaisawasdee et al., 2005; Martínez-Villaluenga et al., 2008; Sun et al., 2016). However, the relatively low stability of the enzyme limits its use in these applications since high temperatures are

required in these industrial processes (Park and Oh, 2010; Vera et al., 2016).

KlβGal, encoded by the *LAC4* gene, belongs to the GH2 family together with a lot of prokaryotic β-galactosidases from organisms like *Escherichia coli* and *Bacillus circulans*. It is remarkable that most of the eukaryotic β-galactosidases are classified in the GH35 family, which suggests a different evolutionary origin between β-galactosidases from *Kluyveromyces* genus and the rest of the eukaryotic enzymes (Pereira-Rodríguez et al., 2012).

The tridimensional structure of the enzyme in crystallization conditions (PDB accession code 3OBA) shows that KlβGal subunits, each of them folded into five domains like other GH2 β-galactosidases (Figure 3A), are arranged into a tetrameric structure (Figure 3B).

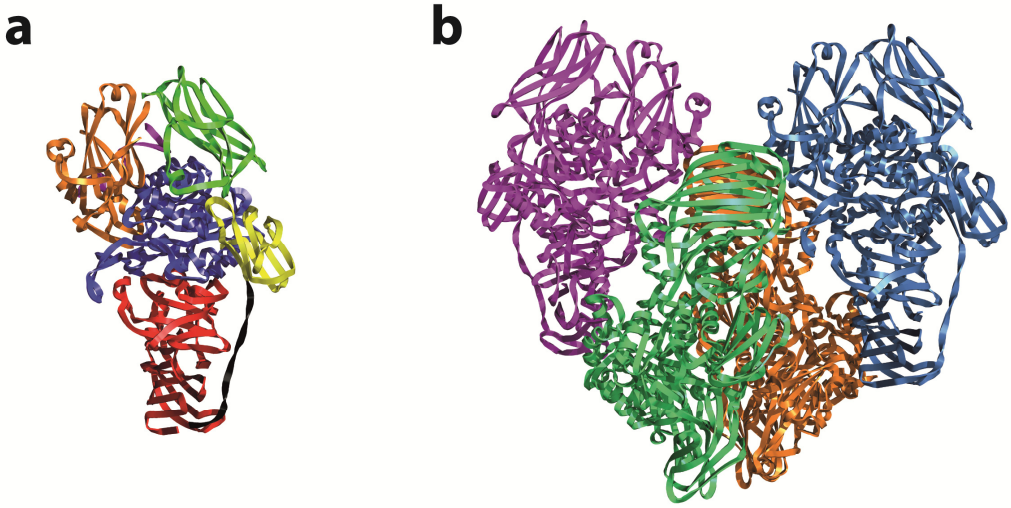

Figure 3: Cartoon representation of monomer (a) and tetrameric structure (b) of KlβGal. a) The five domains of the monomer are represented by different colors (domain 1: orange; domain 2: green; domain 3: blue; domain 4: yellow; domain 5: red). b) The four subunits of the tetramer are represented by different colors (subunit A: green; subunit B: orange; subunit C: purple; subunit D: blue)

The tetramer consists in an assembly of two identical dimers, with higher dissociation energy for the dimers than for their assembly. This characteristic could determine the relative proportion of different oligomeric forms once reached the stationary equilibrium in solution (Pereira-Rodríguez et al., 2012). Previously, it was already observed that KlβGal in solution naturally organizes in several oligomeric forms, from monomers to tetramers. Moreover, enzymatically active forms correspond to dimers or high-order oligomers (Becerra et al., 1998). Therefore, a variation of the dissociation energy of the oligomers might cause differences on the enzymatic activity of KlβGal, due to a change of the relative contribution of different oligomers in the equilibrium.

1.5. Applications

β-galactosidases have been used by food, dairy and fermentation industries in several applications.

One of the well known uses of these enzymes is the removal of lactose from milk products (Neri et al., 2008).

Lactose is a disaccharide that is not well digested by an important proportion of the world population. In most humans, just as it happens with the rest of mammals, after the first months of life, the activity levels of lactase (or lactose-phlorizin hydrolase) diminish to undetectable levels. Therefore, in these people, the ingestion of lactose-containing foods in adults produces lactose accumulation in the large intestine, causing several undesirable effects, such as lactose fermentation by microflora resulting in diarrhea, flatulence and cramps, tissue dehydration due to osmotic effects and poor calcium absorption because of the low acidity (Panesar et al., 2006). Interestingly, some descendants of pastoralist populations maintain

into adulthood high lactase activity levels, which give them the ability to digest milk and other dairy products throughout their lives. This ability is known as lactase-persitence (LP). The frequency of this evolutionary adaptation is different in populations around the world, varying from high rates in North Europe (>90% in Scandinavia and Holland), to very low frequencies in other areas like Asia (≈ 1% in Chinese) (Swallow, 2003). In adults worldwide, the LP frequency is estimated to be 35 per cent (Ingram et al., 2009; Itan et al., 2010).

Hydrolysis of lactose by β-galactosidases makes it possible to prepare milk and other related dairy products with reduced lactose and therefore suitable for consumption by people without LP (Husain, 2010).

Although this application of β-galactosidases is the most extended in food industry, there are other important uses of these enzymes.

Some physicochemical properties of lactose are problematic in candies and fermented dairy products. For example, hygroscopy of this disaccharide results in a strong tendency to absorb flavours and odors, and its low solubility produces many defects in refrigerated dairy products, such as deposit formation, grainy texture and crystallization (Panesar et al., 2006; Panesar et al., 2007). Besides an increase in solubility, hydrolysis of lactose by β-galactosidases improves other sensorial and technological qualities of the products, such as a decrease in the fermentation period and higher sweetness (Dutra Rosolen et al., 2015).

Finally, β-galactosidases are used to hydrolyze lactose in whey, a cheese manufacturing by-product that is an important environmental problem if not properly treated. Besides, the hydrolysis of lactose results in a sweet whey that can be used in the baking, confectionery, dairy and soft drinks

industry, adding value to the bioremediation process (Panesar et al., 2006).

Transgalactosylation reactions of β-galactosidases can also be used to obtain interesting products for the food and pharmaceutical industry. These products are galactooligosaccharides well-known prebiotics with several beneficial biologic effects (see next section).

2. GALACTOOLIGOSACCHARIDES

As a result of transgalactosylation activity, β-galactosidases produce galactooligosaccharides (GOS), also known as trans-galactooligosaccharides (TOS) (Roberfroid, 2007). GOS were defined (Torres et al., 2010) as a series of substances produced from lactose, which include carbohydrates with 2 to 8 saccharide units, characterized by the presence of a terminal glucose and the rest of saccharide units being galactoses, and disaccharides with two galactose units.

Galactooligosaccharides have been recognized as prebiotics. This implies that they have three differential features (Roberfroid, 2007)

- Indigestible nature (resistance to gastric acidity, to hydrolysis by mammalian enzymes and to gastrointestinal absorption)
- They are fermented by gut bacteria
- They promote the growth of specific beneficial (probiotic) intestinal microflora

Diverse oligosaccharides were initially proposed as prebiotics. These include soybean oligosaccharides (SOS), lactosucrose, isomalto-oligosaccharides (IMOS), glucooligosaccharides (GLOS), xylo-oligosaccharides (XOS), mannan oligosaccharides (MOS), gentio-

oligosaccharides (GeOS), pectin-derived acidic oligosaccharides (pAOS), xanthan-derived oligosaccharides (XDOS), arabinoxylan oligosaccharide (AXOS), chito-oligosaccharide (COS), alginate-derived oligosaccharide (ADO) and agarooligosaccharide (AOS) (Kothari et al., 2014). However, nowadays only a few are considered unanimously as prebiotics based on consolidated scientific evidence. GOS, along with inulin type fructans, fructooligosaccharides (FOS), lactulose and human milk oligosaccharides (HMO), belong to this select group (Lamsal, 2012; Rastall, 2010; Roberfroid, 2007). In the last years, due to consumer´s growing attention in the functional foods, GOS are valuable components of verified prebiotic nature.

2.1. Applications

One of the reasons for the commercial interest in GOS is the similarity of these oligosaccharides with those present in the human milk (HMO). HMO have been proposed as components that promote the growth and development of breastfed babies' immune system and microflora. Due to the undesirable differences observed in the gut microflora of infants fed by human and formula-milk, it has been suggested to supply commercial formula with GOS and other oligosaccharides. The restoration of physiological human-milk fed babies indicators after feeding with powdered milk products supplemented with GOS has been reported (Sangwan et al., 2011; Vandenplas et al., 2015).

GOS benefits have been observed not only in infants, but also in adult and elderly people. Several studies have been shown the potential uses of GOS in the treatment or prevention of a variety of disorders, such as the competition of pathogenic organisms with beneficial microflora, allergies

and asthma, and colorectal cancer (Bruno-Barcena and Azcarate-Peril, 2015). Moreover, it has been suggested that they could modulate the immune system, preventing bacterial attachment to colonic epithelium because of their anti-adhesive properties (Lamsal, 2012; Sangwan et al., 2011).

Most of these healthy effects seem to be related with two events triggered by the prebiotics. The first one is the selective proliferation of *Bifidobacterium* and *Lactobacillus* in the gut avoiding the colonization by pathogens. In addition, these beneficial bacteria modulate the immune system.

The second effect is the production of short chain fat acids (SCFA), like acetic propionic and butyric acids. A high SCFA concentration changes colon environment and results in decreased solubility of bile acids, decreased pH, reduced absorption of ammonia and increased mineral absorption (Wong et al., 2006). This in turn promotes beneficial effects, such as reduction of cancer risk and control of cholesterol and serum lipid level (Sangwan et al., 2011).

2.2. Profile of GOS β-galactosidase products

Commercial preparations of GOS contain a mixture of oligosaccharides and variable quantities of other sugars like glucose, galactose and lactose that remain after the catalytic process. The nature and structure of oligosaccharides (linkages of the chain and degree of polymerization) depend on the β-galactosidase used in the synthesis and on the conditions selected in the process (Torres et al., 2010). Considering the ability of some β-galactosidases of producing GOS with different linkage bonds (Gal β(1-2), β(1-3), β(1-4), and β(1-6)) and polymerization (disaccharides,

trisaccharides, tetrasaccharides…), there is a huge number of synthesized GOS depending on the features of the enzyme used (Rodriguez-Colinas et al., 2011) (Table 1).

Table 1: Structural properties of GOS detected in studies with *K. lactis*, *Bacillus circulans* and *A. oryzae* β-galactosidases.

GOS component	Chemical structure	Short name
Disaccharides	Gal-β(1→6)-Glc	allolactose
	Gal-β(1→6)-Gal	6-galactobiose
	Gal-β(1→4)-Gal	4-galactobiose
	Gal-β(1→3)-Glc	3-O-β-galactosyl-glucose
	Gal-β(1→3)-Gal	3-galactobiose
Trisaccharides	Gal-β(1→6)-Gal-β(1→4)-Glc	6´-O-β-galactosyl-lactose
	Gal-β(1→4)-Gal-β(1→4)-Glc	4´-O-β-galactosyl-lactose
	Gal-β(1→3)-Gal-β(1→4)-Glc	3´-O-β-galactosyl-lactose
	Gal-β(1→4)-Gal-β(1→3)-Glc	4´-O-β-galactosyl-3-O-β-galactosyl-glucose
Tetrasaccharides	Gal-β(1→6)-Gal-β(1→6)-Gal-β(1→4)-Glc	6´-O-β-(6-galactobiosyl)-lactose
	Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc	4´-O-β-(4-galactobiosyl)-lactose

Although a lot of β-galactosidases have been tested for the production of GOS (Park and Oh, 2010), food industry tends to use enzymes from GRAS organisms (Mlichová and Rosenberg, 2006) such as *K. lactis*, *Bacillus circulans* and *A. oryzae*. After optimizing reaction conditions for these three enzymes in order to maximize GOS production, some differences were observed in the profile of GOS produced by the enzymes, concerning their bond type and their oligomerization pattern.

By using β-galactosidases from *K. lactis* (Rodriguez-Colinas et al., 2011) and *A. oryzae* (Urrutia et al., 2013), data show a preference in production of trisaccharides like 6-Galactosyl-Lactose, followed by a considerable proportion of disaccharides like allolactose and 6-galactobiose. Moreover,

most of the products are characterized by the presence of β (1-6) bonds; although in fewer amounts, in both cases GOS with β (1-4) and β (1-3) bonds were also detected.

GOS profile produced by β-galactosidase from *B. circulans* is characterized by a major proportion of β (1-4) bonds, being also trisaccharides the most detected oligosaccharides, although the proportion of disaccharides are much lower than the observed in the GOS produced by the other two enzymes (Rodriguez-Colinas et al., 2012).

3. ENZYME ENGINEERING

The use of enzymes in industrial applications is usually limited by the physicochemical properties of the biocatalyst, which must be compatible with the conditions of the industrial process. These industrial conditions tend to be harsher than the conditions of the natural host of the enzyme. In order to improve the performance of natural biocatalysts in these industrial applications, traditionally physical methods like immobilization and embedding were implemented. However, these procedures achieve a limited improvement of the catalytic properties of the enzymes. The huge progress of the DNA recombinant techniques in the last decades allowed the development of specific tools to improve the enzyme properties by genetic modifications (Davids et al., 2013; Yang et al., 2014).

3.1. Strategies

Several strategies were designed in order to obtain improved mutant enzymes. They can be classified in three general groups: directed evolution, semi-rational approaches and rational approaches.

Directed evolution has been extensively used to change enzyme properties (Cobb et al., 2012). The process does not require previous structural or functional knowledge of the enzyme and mimics the natural selection in laboratory conditions, reducing evolution time to weeks or months (Joshi and Satyanarayana, 2015). To achieve that goal, these methods require iterative rounds of generation of genetic diversity followed by screening. To create genetic diversity, methods like DNA shuffling, use of a mutator strain, chemical mutagenesis or error-prone PCR were used. In spite of the potential of this group of techniques, they are limited by the large size of the generated library, which complicates the screening step and implies the development of powerful selection methods in order to explore all the genetic diversity generated (Eriksen et al., 2014).

Due to the known structural and biochemical data of progressively more and more enzymes, rational approaches that need prior knowledge of the proteins are increasingly used in the last years. One of the most used rational methods, site-directed mutagenesis, is based on the analysis of structural and functional properties of the enzyme and the selection, generally by computational methods, of a residue, to be mutated in order to change enzyme features. The advantages of this method are its high efficiency and that it does not require a strong screening step, such as it occurs in random approaches (directed evolution) (Yang et al., 2014).

Finally, semi-rational methods like Iterative Saturation Mutagenesis (ISM) combine directed evolution with rational approaches, in order to create

small focused libraries in specific points or areas of the enzyme previously selected on a rational base (Davids et al., 2013).

3.2. Stability improvement of enzymes by protein engineering

Enzyme stabilization is not only desirable for the adaptation of the protein to the industry processes, it is also interesting for obtaining new variants able to better support additional mutations. On the native form, some mutations destabilize the enzyme, but the achievement of a more stable protein may reduce this effect and increase the evolutionary options. Therefore, a wider range of new properties could be accomplished by mutagenesis of this new enzyme variant (Bloom et al., 2006; Brown et al., 2010).

The principles that guide the attainment of stability in proteins are not fully understood yet (Iyer and Ananthanarayan, 2008). This has made that random or directed evolution approaches have been extensively used, obtaining in some cases noticeable results (Singh et al., 2013). However, structural studies of thermophilic enzymes in comparison with other mesophilic counterparts have given some insights into the structural keys, which make the enzymes more stable. In general, thermostability is a result of the presence of some amino acids in important protein areas, whose unfolding is limiting in the protein denaturation process, and which confer a more rigid conformation and higher hydrophobic contacts to the protein (Van den Burg et al., 1998; Veltman et al., 1996; Vieille and Gregory Zeikus, 1996). Moreover, some thermal fluctuation in specific flexible residues of the protein surface are thought to unleash unfolding; therefore mutation of these amino acids has more potential to improve the enzyme thermostability (Li and Cirino, 2014). Keeping in mind these

principles, several strategies have been developed to attempt the stabilization of an enzyme from a rational perspective.

To increase the rigidity of enzymes, one of the most utilized methods is the introduction of new molecular interactions such as disulfide bonds, chemical crosslinks and salt bridges, especially in flexible areas like surface turns and loops (Singh et al., 2013). Moreover, the higher rigidity and stability of multimeric enzymes in comparison to the corresponding monomeric structures (Iyer and Ananthanarayan, 2008) has encouraged the stabilization of multimeric enzymes by preventing the subunit dissociation with the introduction of inter-subunits disulfide bonds and other strategies (Fernandez-Lafuente, 2009). The recent development of new computational tools makes it possible to detect key residues and areas, and increases the success rate of these rational approaches (Li and Cirino, 2014).

3.3. Catalytic modifications by enzyme engineering

Modification of the enzymatic catalytic properties is one of the most searched goals for enzyme engineers. Changes in activity, specificity, enantioselectivity and even design and evolution of new enzyme activities by expanding substrate acceptance have been achieved for several enzymes (Otten et al., 2010; Reetz et al., 2005; Tiwari, 2016). In contrast with stability improvement approaches, changes in catalytic features tend to be obtained by mutation of residues in catalytic or substrate binding sites, or in their proximity (Jochens et al., 2011; Tiwari, 2016).

One of the catalytic activities that have been pursued to maximize in some glycosyl-hydrolases (GH) is the transglycosylation activity, by lowering the H/T (hydrolysis vs transglycosylation activity) ratio of the enzymes.

Structural studies of transglycosidases (GH with dominant transglycosylation activity) and increased transglycosylation mutants show some important structural keys that could confer transglycosylation features to GH. Thus mutations that produce i) destabilization of the hydrogen bonding network in negative subsites ii) increased affinity with acceptor sugar in positive subsites iii) lower affinity for catalytic water, tend to maximize synthesis of oligosaccharides (Bissaro et al., 2014). The creation of a more hydrophobic environment in the active site of the enzyme also helps for obtaining enhanced transglycosylation variants, by promoting the binding of the sugar acceptors and the exclusion of water molecules (Abdul Manas et al., 2015; Yin et al., 2009).

4. IMMOBILIZATION

Apart from enzyme engineering by genetic modifications, immobilization techniques have been used to improve enzyme characteristics in order to adapt them to industrial process conditions.

Immobilization can improve several enzyme features like activity, thermal stability, solvent stability, selectivity and substrate tolerance. Moreover, co-immobilization allows multi-step reactions (Singh et al., 2013). Furthermore, this type of techniques allows enzyme reuse and facilitates separation from product, which involves important savings in the industrial procedure (Polizzi et al., 2007).

The main immobilization strategies are entrapment, encapsulation, attachment to solid support and self-immobilization (Brady and Jordaan, 2009).

Different materials to immobilize enzymes have been used, including polymers like alginate, chitosan, collagen or sepharose, and inorganic materials like zeolites, ceramics, glass or activated coal (Datta et al., 2012). β-galactosidases have been immobilized by diverse strategies. Focusing on the β-galactosidase from *K. lactis*, it has been immobilized in supports like thiosulfinate/thiosulfonate (Ovsejevi et al., 1998), cotton fabric (Li et al., 2007), magnetite covered by polysiloxane–polyvinyl alcohol (Neri et al., 2008), silicon dioxide nanoparticles (Verma et al., 2012), polymeric ultrafiltration membranes (Güleç, 2013) or chitosan microparticles (Klein et al., 2013).

In this thesis, the study of structural features of AnβGal and KlβGal serves as a starting point for the engineering of these two β-galactosidases, in order to improve their properties for their utilization in applications like synthesis of GOS. Moreover, preliminary immobilization studies with mutant and native KlβGal were carried out.

Objectives

The aim of this thesis is to study and improve by rational mutagenesis the features of two industrial enzymes, *Kluyveromyces lactis* β-galactosidase and *Aspergillus niger* β-galactosidase. To achieve this goal, the thesis addresses the following specific objectives:

1. Expression, purification and crystallization of the β-galactosidase from *Aspergillus niger*.
2. Structural characterization of β-galactosidase from *Aspergillus niger*.
    a. Structural studies of the free enzyme
    b. Structural studies of the enzyme in complex with natural substrates
    c. Obtaining mutant enzymes with increased transgalactosylation and hydrolytic activity
3. Improvement of *Kluyveromyces lactis* β-galactosidase for high-temperature industrial applications by rational mutagenesis.
4. Preliminary immobilization study of native and obtained thermostable mutant β-galactosidase from *Kluyveromyces lactis*.

Outline of this thesis

β-galactosidases are extensively used by food industry in a number of applications. However, their use as biocatalysts is limited because of the particular conditions required in some of these procedures, which tend to be different to the optimal catalytic requirements of the enzymes. Besides, usually catalytic features of the enzyme do not completely fit with the objectives of the industrial process.

This thesis deals with the structural study and engineering of these enzymes towards more suitable forms for industrial applications. β-galactosidases from the filamentous fungus *Aspergillus niger* and from the yeast *Kluyveromyces lactis,* two of the most used on food industry, are studied.

In the first chapter we describe the expression of the β-galactosidase from *Aspergillus niger* in *Saccharomyces cerevisiae*, as well as its purification and optimization of the crystallization process. Preliminary X-ray data collected from the diffraction of the better protein crystals obtained is also reported.

In Chapter 2 we expose the structural characterization of the β-galactosidase from *Aspergillus niger*. We have solved the 3-dimensional structure in native form and in complex with several natural substrates. The study of the different interactions with substrates and properties of the catalytic site of the enzyme was reinforced by mutagenesis approaches. This knowledge and the structural comparison between the enzyme and other β-galactosidases of its family allowed to obtain by site-directed mutagenesis new β-galactosidase variants with interesting features for their use in industry.

Chapter 3 shows the rational construction by mutagenesis methods of thermostable variants of the β-galactosidase from *Kluyveromyces lactis*. On the basis of a strategy that aimed to reinforce the quaternary structure of the enzyme by the introduction of new disulfide contacts in subunit interfaces, we also describe the functional properties of the mutants obtained. Finally, we test the convenience for their utilization in some industrial applications like galactooligosaccharides synthesis, and discuss the possible process benefits that could provide.

In last chapter we describe the immobilization of the β-galactosidase from *Kluyveromyces lactis* and one of its previously obtained mutants in two magnetic supports, and we compare the functional properties of immobilized proteins to test the suitability of enzyme variants and the interactions with supports, which might be used in industrial applications.

Chapter 1

Crystallization and preliminary X-ray diffraction data of β-galactosidase from *Aspergillus niger*

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SUMMARY

β-galactosidase from *Aspergillus niger* (AnβGal), belonging to the family 35 glycoside hydrolases, hydrolizes the β-galactosidase linkages in lactose and other galactosides. It is extensively used in the industry owing to its high hydrolytic activity and safety. The enzyme has been expressed in yeasts and purified by immobilized metal-ion affinity chromatography for crystallization experiments. The recombinant AnβGal, deglycosilated to avoid heterogeneity of the sample, has a molecular mass of 109 kDa. Rod-shaped crystals grew with PEG 3350 as main precipitant agent. A diffraction data set was collected to 1.8 Å resolution.

1. INTRODUCTION

The enzyme β-D-galactosidase (EC 3.2.1.23) catalyses the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. It has been mainly used for the hydrolysis of lactose in milk and other dairy products. Furthermore, β-galactosidases have transgalactosylation activities that make them very attractive for the production of prebiotic galactooligosaccharides (GOS). The β-D-galactosidase from the fungus *Aspergillus niger* (AnβGal) is one of the most used enzymes in food industry. Its high optimal temperature allows its use up to 323 K (Panesar et al., 2006), which makes it very valuable for some industrial applications as the synthesis of GOS. Moreover, reported work (Dragosits et al., 2013) has showed that its enzymatic activity is higher than the corresponding to other β-galactosidases from the *Aspergillus* genus. It also seems to tolerate fairly high amounts of organic solvents, which is an important trait in some transglycosylation-based applications using antibiotics or PEG (Dragosits et

al., 2013). Furthermore, *A. niger* is an organism that has been designated GRAS (generally recognized as safe) by the American Food and Drug Administration (Schuster et al., 2002). Unfortunately, the too acidic AnβGal optimum pH largely limits its applications in milk and neutral sweet cheese-whey, derived from hard cheese manufacturing (Rubio-Texeira, 2006).

Despite AnβGal being widely used in the dairy industry, its three-dimensional structure has not been solved. AnβGal encoded by the gene *LACA* belongs to the family 35 of glycosylhydrolases (GH35), which contains most of the eukaryotic β-galactosidases. The three-dimensional structures of those from *Penicillium* sp., *Trichoderma reesei*, *Aspergillus oryzae* and *Homo sapiens* have been previously reported (Maksimainen et al., 2011; Maksimainen et al., 2013; Ohto et al., 2012; Rojas et al., 2004).

The knowledge of the molecular structure of AnβGal is necessary to fully understand its particular enzymatic activities and to improve its biotechnological potential. In this study, we describe the purification protocol used to overproduce the enzyme in *Saccharomyces cerevisiae*, the crystallization of its deglycosylated form and a preliminary X-ray crystallographic analysis.

2. MATERIALS AND METHODS

2.1. Macromolecule production

The *LACA* gene was amplified by PCR and cloned by homologous recombination in *Saccharomyces cerevisiae*. The 30 first nucleotides of the sequence (signal peptide) were not cloned. The final construct was expressed grown in 400 ml YPHSM medium (1,5% w/v glucose, 3% v/v glycerol, 1% w/v yeast extract and 8% w/v peptone) at 303 K and 250 rpm for 72 h in a 2 L Erlenmeyer flask. Cells were collected by centrifugation (11,800 g for 10 min at 277 K) and the extracellular medium was filtered through a 0.45µm disposable syringe filter (Macherey-Nagel). The collected supernatants were applied to an affinity chromatography system in a HisTrap HP 5 mL column, coupled to an Äkta purifier (GE Healthcare) that had been equilibrated with buffer A (100 mM sodium phosphate buffer, 500 mM NaCl, 25 mM imidazole, pH 7). The column was equilibrated in buffer A, and after sample injection, it was washed with 10 x column volumes of the same buffer. Then, the protein was eluted with 25 mM imidazole in buffer B (100 mM sodium phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7). The protein solution was concentrated to 2 mL by ultrafiltration with Amicon Ultra-4 (Millipore, UFC803024). The homogeneity of the purified protein was evaluated by SDS–PAGE (Laemmli, 1970). Macromolecule production details are given in Table 1.

Table 1: AnβGal production information.

Primers carry several nucleotides (shown in bold) of the specific sequence required for homologous recombination with the vector YEpFLAG, and a 6xHis sequence for introduction of a C-terminal tag (shown in italics).

Source organism	*Aspergillus niger*
DNA source	pVK1.1 plasmid
Forward primer	**CCAGCATTGCTGCTAAAGAAGAAGGGGTACCTTTGGATAAAAGA**TCCATT AAGCATCGAATCAAT
Reverse primer	**GGATCCATCGATAGATCTCCCGGGCTCGAGCTA***ATGATGATGATGATGAT G*GTATGCACCCTTCCGCTT
Cloning vector	YEpFLAG-1
Expression vector	YEpFLAG-1
Expression host	BJ3505 *Saccharomyces cerevisiae* (*pep4::HIS3*, *prb-Δ1.6R HIS3*, *lys2-208*, *trp1- Δ101*, *ura 3–52*, *gal2*, *can1*)

2.2. Crystallization

Prior to crystallization experiments, glycan chains were removed using endoglycosidase H (Endo H; New England BioLabs) under native conditions. Endo H treatment was carried out for 3 h following the manufacturer's instructions. An extra purification step using a gel filtration column (HiLoad™ 16/60 Superdex™ 200 prep grade column, GE Healthcare) was performed after deglycosylation in order to eliminate Endo H contamination from the protein sample. The deglycosylated protein was concentrated to 1.5 mg/mL by ultrafiltration using Amicon Ultra-4 (Millipore, UFC 803024).

Crystallization conditions were initially explored using commercially available screens. PACT Suite and JCSG+ Suite from Qiagen were assayed using the sitting-drop vapor-diffusion method at 291 K. Drops consisting of 0.5 µL precipitant and 0.5 µL pure AnβGal (1.5 mg/mL in 150 mM NaCl, 50 mM Tris–HCl pH 7.5) were equilibrated against 65 µl reservoir solution on sitting-drop microplates. Crystals grew in several conditions with polyethylene glycol (PEG) 3350 as the main precipitant agent. Optimization of the crystallization conditions was performed through further sitting-drop experiments. In them, the same volume of protein solution and precipitant (1 µL) were mixed and equilibrated against 200 µl reservoir solution on sitting-drop MRC Maxi 48-well Crystallization Plate (Hampton Research) (Table 2).

Table 2: Crystallization

Method	Vapour diffusion, sitting drop
Plate type, screening	Innovaplate SD-2
Plate type, optimization	Maxi 48-well Crystallization Plate
Temperature (K)	291
Protein concentration (mg/mL)	1.5
Buffer composition of protein solution	150 mM NaCl, 50 mM Tris–HCl pH 7.5
Composition of reservoir solution	21-24% (w/v) PEG3350, 200 mM Li_2SO_4 and 100 mM BisTris pH 5.5-6.0
Volume and ratio of drop	2 µL; 1:1 ratio
Volume of reservoir (µL)	200

2.3. Data collection and processing

Crystals were soaked in precipitant solution containing an additional 20% (w/v) glycerol (Garman and Mitchell, 1996) a few seconds before being flash-cooled to 100 K. Diffraction data were collected using synchrotron radiation. The diffraction data collected were processed with iMOSFLM (Battye et al., 2011) and AIMLESS (Evans, 2006) as distributed in the CCP4 suite (Winn et al., 2011). Data collection statistics are summarized in Table 3.

Table 3: Data collection and processing. Values for the outer shell are given in parentheses.

Diffraction source	ALBA beamline XALOC
Wavelength (Å)	0.97947
Temperature (K)	100
Detector	PILATUS
Crystal-detector distance (mm)	293.12
Rotation range per image (°)	0.2
Total rotation range (°)	200
Exposure time per image (s)	0.2
Space group	$P\ 2_1 2_1 2_1$
a, *b*, *c* (Å)	85.57, 111.42, 126.73
Mosaicity (°)	0.52
Resolution range (Å)	42.79–1.80 (1.84–1.80)
Total No. of reflections	1160311 (49134)
No. of unique reflections	173058 (7448)
Completeness (%)	99.9 (99.9)
Redundancy	6.7 (6.6)
$\langle I/\sigma(I)\rangle$	8.8 (4.4)
$CC_{1/2}$	99.1 (68.5)
R_{meas}†	0.13 (0.54)
Overall *B* factor from Wilson plot ($Å^2$)	8.3

† R_{meas}: - multiplicity-independent R = ∑ (Sqrt(N/(N-1))(|Ihl - <Ih>|))/∑ (<Ih>)

3. RESULTS AND DISCUSSION

AnβGal is an extracellular highly glycosylated enzyme, its molecular weight dropping more than 50% after treatment with endoglycosidase H (Endo H) (Figure 1). Moreover, these glycosylations make the protein very heterogeneous, which usually hinders the crystallization process. To remove this obstacle, the protein sample was treated with Endo H prior to the crystallization step (Lehle et al., 2006). Endo H cleaves asparagine-linked oligo-mannoses generating a truncated sugar moiety with one N-acetylglucosamine residue remaining on the glycosylation site.

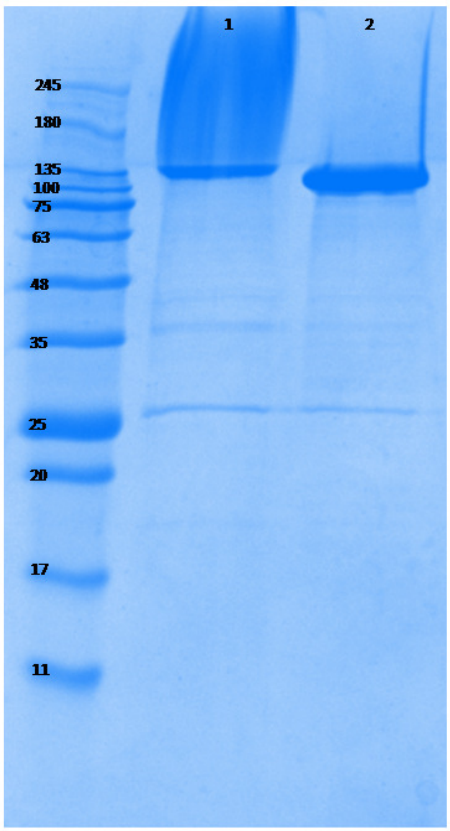

Figure 1: SDS–PAGE analysis of glycosylated (lane 1) and deglycosylated (lane 2) purified AnβGal. The glycosylated sample shows a wide smeared pattern with an average molecular weight of 200 kDa. The deglycosylated sample shows a molecular weight of 109 kDa as predicted from the amino-acid sequence.

Initial screenings using PACT Suite and JCSG+ Suite with the purified deglycosylated AnβGal gave needles in some conditions of the JCSG+ Suite, which contain PEG 3350 as the main precipitant agent. Optimization was assayed by sampling protein and precipitant concentration, and by varying pH. Best rod-shaped crystals grew in 21 days from 21-24% (w/v) PEG3350, 200 mM Li_2SO_4 and 100 mM BisTris pH 5.5 – 6.0 (Figure 2A).

A full data set was collected at 100 K on the XALOC beamline at ALBA station (Cerdanyola del Vallès, Spain). The crystals belong to space group $P2_12_12_1$, as observed from the systematic absences, with unit-cell parameters a = 85.57, b = 111.42, c=126.73 Å, and diffracted up to 1.8 Å resolution (Figure 2B, Table 3). As calculated from its sequence analysis, the molecular mass of the monomer is 109 kDa. The Matthews coefficient value of 2.77Å ^{3} Da^{-1} (Matthews, 1968) corresponding to 56% solvent content reveals the presence of one molecule in the asymmetric unit. Structure determination by molecular replacement is in progress using the coordinates from *Aspergillus oryzae* β-galactosidase (PDB code 4IUG) (Maksimainen et al., 2013), which shows 75% sequence identity. It is hoped that analysis of the structure will give insights into the mechanism and specificity of AnβGal, which is most useful for biotechnological purposes.

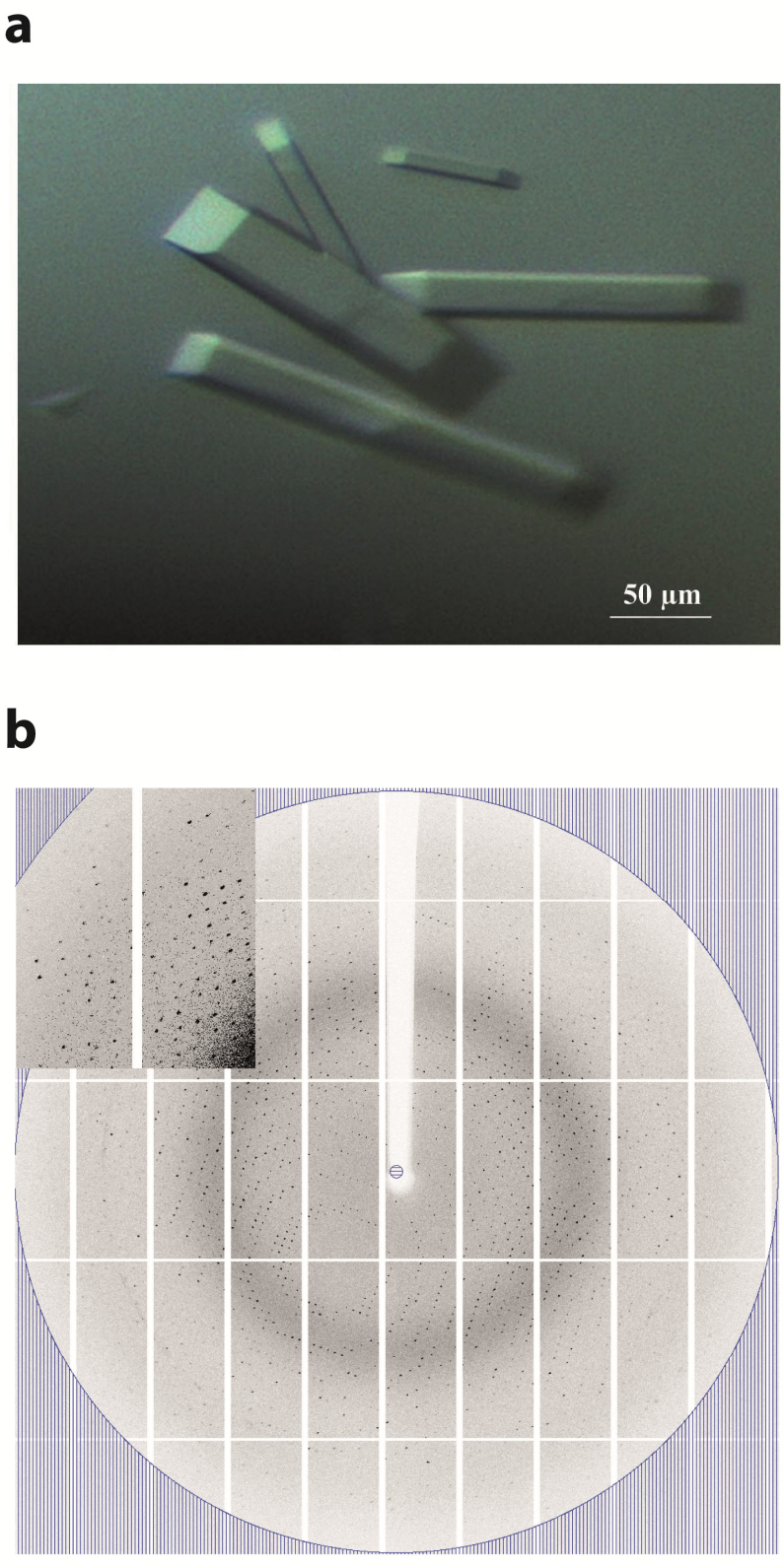

Figure 2: (a) Crystals of AnβGal grown in 21–24%(w/v) PEG 3350, 200 mM Li_2SO_4, 100 mM bis-tris pH 5.5. (b) X-ray diffraction pattern obtained using a synchrotron source. The outer circle corresponds to 1.8 Å resolution.

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Chapter 2

Structural, mutagenesis and biochemical analysis of β-galactosidase from *Aspergillus niger* gives insights into specificity preferences of GH35 β-galactosidases

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(In revision)

SUMMARY

β-galactosidases are biotechnologically interesting enzymes that catalyze the hydrolysis or transgalactosylation of β-galactosides. Among them, the *Aspergillus niger* β-galactosidase (AnβGal) belongs to the glycoside hydrolase family 35 (GH35) and is widely used in the industry due to its high hydrolytic activity degrading lactose. We present here its three-dimensional structure in complex with different oligosaccharides, to illustrate the structural determinants of the broad specificity of the enzyme against different glycoside linkages. Remarkably, the residues Phe264, Tyr304 and Trp806 make a dynamic hydrophobic platform that accommodates the sugar at subsite +1 suggesting a main role on the recognition of structurally different substrates. Moreover, complexes with the trisaccharides show two potential subsites +2 depending on the substrate type. This feature and the peculiar shape of its wide cavity suggest that AnβGal might accommodate branched substrates from the complex net of polysaccharides composing the plant material in its natural environment.

Relevant residues were selected and mutagenesis analyses were performed to evaluate their role in the catalytic performance and the hydrolase/transferase ratio of AnβGal. Thus, we generated mutants with improved transgalactosylation activity. In particular, the variant Y304F/Y355H/N357G/W806F displays a higher level of galacto-oligosaccharides (GOS) production than the *Aspergillus oryzae* β-galactosidase, which is the preferred enzyme in the industry owing to its high transferase activity. Our results provide new knowledge on the determinants modulating specificity and the catalytic performance of

fungal GH35 β-galactosidases. In turn, this fundamental background gives novel tools for the future improvement of these enzymes, which represent an interesting target for rational design.

1. INTRODUCTION

β-D-galactosidases are enzymes widely used in the food industry, mainly in the dairy sector. They catalyze the hydrolysis of terminal nonreducing β-D-galactose residues in β-D-galactosides by breaking galactosyl bonds. This hydrolytic activity has been used to degrade lactose of dairy products like milk or cheese whey in its two components, glucose and galactose (Oliveira et al., 2011). This makes possible the production of lactose-free products, a growing market because of the high percentage of lactose malabsorbers, which represents most of the population in regions like Asia or Africa (Itan et al., 2010; Swallow, 2003).

In addition to its hydrolytic activity, β-galactosidases can perform transglycosylation depending on the conditions (Oliveira et al., 2011). Thus, when a high concentration of a substrate like lactose is present in the medium, these enzymes may transfer a galactose unit to the substrate generating a longer product. Therefore, transgalactosylation produces oligosaccharides with a higher degree of polymerization, called galacto-oligosaccharides or GOS (Gosling et al., 2010). GOS are prebiotics, indigestible components that promote growing of beneficial microflora within the gut. Their similarities with the human milk oligosaccharides have recently attracted a great attention. Some studies support that they promote health benefits and they are used in several applications such as infant nutrition, growing up milk, dairy products, beverages, clinical nutrition, bakery, and pet food (Sangwan et al., 2011).

Although bacteria could offer more versatility, the corroborated GRAS (Generally Recognized As Safe) status of yeasts like *Kluyveromyces lactis* and *Kluyveromyces marxianus*, and fungi like *Aspergillus niger* and *Aspergillus oryzae*, still place them among the favorite sources of enzymes for food and pharmaceutical industry (Rubio-Texeira, 2006). In particular, β-D-galactosidase from *A. niger* (AnβGal) is used for the hydrolysis of acid whey, which derives from the production of fresh or soft cheeses (Yang and Silva, 1995). Although it was demonstrated that AnβGal has some transgalactosylation activity (Dragosits et al., 2014; Toba and Adachi, 1978), its high hydrolytic ability makes it not suitable for GOS synthesis applications.

AnβGal belongs to glycoside hydrolases family 35 (GH35). Several eukaryotic β-galactosidases of this family have been structurally characterized. The enzymes from *Penicillium* sp. (Rojas et al., 2004), *Trichoderma reesei* (Maksimainen et al., 2011), *Homo sapiens* (Ohto et al., 2012), *Aspergillus oryzae* (Maksimainen et al., 2013) and *Solanum lycopersicum* (PDB ID 3W5F, not published) have been previously reported. AnβGal presents more than 70% sequence identity with the β-galactosidases from *Penicillium* sp. and *A. oryzae*. However, and in contrast to AnβGal, it was described that these two last enzymes have high transgalactosylation activity (In and Chae, 1998; Iwasaki et al., 1996; Neri et al., 2009). In fact, a comparison between β-galactosidases from *A. oryzae* and *A. niger*, showed clearly differences in the relative levels of hydrolysis and transferase activities (Guerrero et al., 2015), in spite of their high sequence similarities.

Among the fungal GH35 β-galactosidases reported structures, none of them shows the binding mode of natural substrates at the active site.

Those complexes with a galactose molecule represent the product that would remain bound at the catalytic pocket after hydrolysis of a β-galactoside. The knowledge of the binding mode of longer molecules with two or three sugar units could give us insights into the factors and structural elements that define the catalytic activity of the enzyme.

In this study, we report the structure of AnβGal and also its inactivated E298Q mutant in complex with the oligosaccharides allolactose, 3-galactosyl-glucose, 6-galactosyl-galactose, 4-galactosyl-lactose and 6-galactosyl-lactose, which are potential substrates (Sykes et al., 1983) or products (Urrutia et al., 2013) of the enzyme. Mapping of the catalytic pocket of the complexes and comparison between AnβGal and other fungal GH35 β-galactosidase structures allowed us to uncover possible key residues involved in hydrolysis and transfer activity, which was verified by the generation of mutant variants and their subsequent study. The results obtained give us valuable information about the mechanisms that define the catalytic activity of fungal GH35 β-galactosidases. Moreover, we enhanced some of the catalytic features of AnβGal, which could be extended to other GH35 β-galactosidases in order to achieve mutant variants more suitable for their use in industrial applications.

2. MATERIAL AND METHODS

2.1. Cloning, expression and purification

LACA gene encoding a β-galactosidase from *A. niger* (AnβGal) was amplified from the pVK1.1 plasmid and cloned into the YEpFLAG vector by homologous recombination in the yeast strain BJ3505 (*pep4::HIS3*, *prb-Δ1.6R HIS3*, *lys2-208*, *trp1-Δ101*, *ura 3–52*, *gal2*, *can1*; Eastman Kodak Company). N-terminal FLAG peptide tag of the plasmid was substituted by a 6xHis TAG sequence. The signal peptide of the protein (30 first nucleotides of the gene) was not cloned.

Wild type AnβGal and its mutants were expressed in BJ3505 *Saccharomyces cerevisiae* strain (Kodak). Enzymes were purified with HisTrap HP columns of 5 ml (GE Healthcare), on a protein liquid chromatography system (ÄKTA FPLC; GE Healthcare), as described previously (Rico-Diaz et al., 2014).

In order to perform the crystallization experiments, native AnβGal and inactive mutant AnβGal-E298Q samples were deglycosylated with endoglycosidase H (Endo H, New England Biolabs) and an additionally step of purification using a gel filtration column (HiLoad 16/60 Superdex 200 prep-grade column, GE Healthcare) was performed. Protein samples were subsequently concentrated using a 30 kDa cutoff membrane.

2.2. Mutagenesis

AnβGal mutant variants E298Q, Y304F, Y304A, W806F, W806S, Y304F/Y355H/N357G and Y304F/Y355H/N357G/W806F were obtained by PCR, using commercial kit QuikChange Lightning (Stratagene). Oligonucleotide design and mutagenic procedures were performed following

the manufacturers' recommendations.

Purifications of mutant AnβGal variants were performed in the same way as wild type AnβGal (see above) but samples not used in crystallization experiments (all mutant variants except E298Q) were not deglycosylated.

2.3. Crystallization and data collection

Crystallization of AnβGal and mutant AnβGal-E298Q (both 1.5 mg ml^{-1} in 150 mM NaCl, 50 mM Tris–HCl pH 7.5) was performed on Cryschem (Hampton Research) sitting drop plates as described previously (Rico-Diaz et al., 2014). Rod-shaped crystals grew from both samples in 21–28% (w/v) PEG 3350, 200 mM Li_2SO_4 and 100 mM Bis-Tris pH 5.5–6.0. For data collection, native crystals were transferred to cryoprotectant solutions consisting of mother liquor plus 20% (v/v) glycerol before being cooled in liquid nitrogen.

Complexes with the natural substrates allolactose, 3-galactosyl glucose, 6-galactosyl galactose, 4-galactosyl lactose and 6-galactosyl lactose (Carbosynth) were obtained from the inactivated mutant by the soaking method (Hassell et al., 2006). To minimize crystal manipulation, drop solution was substituted with a stabilizing solution (24-30% PEG 3350, 100 mM Bis-Tris pH 6, 200 mM Li_2SO_4) containing the substrate at 30 mM concentration and incubated for several days. Then, crystals were soaked in cryoprotectant solution before being frozen in liquid nitrogen. At first, crystals were soaked in stabilizing solution plus 20% (w/v) glycerol but only a molecule of the crioprotectant was found at the active site pocket and, therefore, other compounds were tried. Final cryoprotectant solutions used with crystals from each complex are summarized in Table 1.

Diffraction data were collected using synchrotron radiation at the XALOC beamline at ALBA (Cerdanyola del Vallès, Spain). Diffraction images from native enzyme were processed with iMosfilm (Battye et al., 2010) while those from the complexes were processed using XDS (Kabsch, 2010). All of them were merged using AIMLESS from the CCP4 package (Winn et al., 2011). A summary of data collection and data reduction statistics for all the crystals is shown in Table 1.

2.4. Structure Solution and Refinement

The structure of AnβGal was solved by molecular replacement using the MOLREP program (Vagin and Teplyakov, 2010). The best model was the β-galactosidase from *A. oryzae* (PDB code 4IUG), having 71% identity (81% similarity), from which a template was prepared using the program Chainsaw (Stein, 2008) and a protein sequence alignment of AnβGal onto the template. A solution containing one molecule in the asymmetric unit was found using reflections up to 2.5 Å resolution range and a Patterson radius of 40 Å, which after rigid body fitting led to an *R*-factor of 49%. Crystallographic refinement was performed using the program REFMAC (Murshudov et al., 1997) within the CCP4 suite with flat bulk-solvent correction, maximum likelihood target features. Free *R*-factor was calculated using a subset of 5% randomly selected structure-factor amplitudes that were excluded from automated refinement. Extensive model building using the program COOT (Emsley and Cowtan, 2004) was combined with several rounds of refinement leading to a model showing a continuous density for the whole polypeptide chain. At the later stages, carbohydrates and water molecules were included in the model, combined with more rounds of restrained refinement that led to a final *R*-factor of

15.02 (*R*free 17.14) for all data set up to 1.71 Å resolution. Refinement parameters are reported in Table 1. The structure of E298Q mutant complexed with 6GalGal, showing a different space group, was solved by molecular replacement using MOLREP and the coordinates of native AnβGal as the search model. A first cycle of refinement was performed using REFMAC, combined with model building with COOT. The structures of other AnβGal-E298Q complexes were solved by difference Fourier synthesis using the refined coordinates of the native or mutant form, depending of their space group. Then, coordinates for the ligands were taken from the Protein Data Bank or constructed with GLYCAM server, and manually built into the electron density maps and refined similarly to that described above, to reach the *R*-factors listed in Table 1. Stereochemistry of the models was checked with PROCHECK (Laskowski et al., 1993) and the figures were generated with PyMOL (DeLano, 2002). Root mean square deviation analysis was made using the program SUPERPOSE within the CCP4 package (Winn et al., 2011).

2.5. Kinetics

Kinetic characterizations of AnβGal and their mutants were performed by measuring the glucose produced by the enzyme at different lactose concentrations. Purified samples were diluted on citrate-phosphate buffer pH 3.5. Initial velocity was measured in triplicate with 2-40 μg ml^{-1} of enzyme, depending of the characteristics of the AnβGal variant tested, and using 10, 20, 40, 80 and 160 mM lactose. The reaction time was 3-6 min and the reaction temperature was 40 ºC. The reaction was stopped by heating samples at 96 ºC during 5 min.

Glucose concentration was measured using the commercial kit D-Glucose GOD-POD (Nzytech), following the manufacturers' recommendations. Non-linear fitting using least squares was performed to infer the apparent enzymatic kinetic parameters from Michaelis-Menten plots using Prism 6 (*GraphPad Software Inc.*). Kcat values were calculated assuming a protein molecular mass of 109 kDa.

2.6. Galacto-oligosaccharides (GOS) measurement

GOS and lactose concentrations were determined by HPLC (HPLC Waters Breeze I), using a Waters Sugar-Pak column eluted at 90 ºC with Milli-Q water at a flow rate of 0.5 ml min^{-1}, and a refractive-index detector (Waters 2414).

Reactions were performed by mixing 2 enzyme units (1 U = amount of enzyme capable of liberating 1 mmol of glucose per min under experiment conditions) of pure protein in 0.1 M citrate-phosphate buffer pH 3.5 supplemented with 40% lactose. 750 µL of sample were incubated at 40 ºC and at 250 rpm. Samples were taken at different times: 0, 3, 6, 16, 24, 31 and 48 hours.

Quantification of carbohydrates was performed by external calibration using standard solutions of galactose, glucose, lactose, raffinose and stachyose.

Integration and quantification of data were performed with Breeze II Software (Waters).

Statistical significant differences ($p>0.05$ and $p>0.01$) of GOS yield between wild type AnβGal and the mutants were tested by using a two-tailed Student´s test. Statistical analysis and figures were generated using Microsoft Office Excel 2007.

Table 1: Crystallographic statistics (Values in parentheses are from the high resolution cell).

TABLE 1						
Crystal data	AnβGal	AnβGal-E298Q /Allolactose	AnβGal-E298Q /3-Galactosyl-Glucose	AnβGal-E298Q /6-Galactosyl-Galactose	AnβGal-E298Q /4-Galactosyl-Lactose	AnβGal-E298Q /6-Galactosyl-Lactose
Cryoprotectant	20% (v/v) glycerol	25% (v/v) DMSO	70% (v/v) Paratone 30% (v/v) Paraffin	20% (w/v) PEG 400	10% (v/v) DMSO	10% (v/v) DMSO
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_1$	$P2_1$	$P2_1$
Unit cell parameters						
a (Å)	85.57	84.14	87.13	58.10	59.63	58.28
b (Å)	111.42	111.41	111.64	106.39	106.66	105.87
c (Å)	126.73	125.86	125.80	83.72	84.42	83.72
β				99.18	96.13	99.24
Data collection						
Beamline	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)
Temperature (K)	100	100	100	100	100	100
Wavelength (Å)	0.97948	0.97872	0.97872	0.97949	1.12716	1.12714
Resolution (Å)	40.54-1.71 (1.74-1.71)	46.61-2.40 (2.48-2.40)	47.00-2.45 (2.54-2.45)	46.06-2.27 (2.34-2.27)	46.16-2.30 (2.38-2.30)	46.11-2.15 (2.21-2.15)
Data processing						
Total reflections	889284 (40056)	320992 (31824)	298641 (32454)	318121 (28354)	315538 (30309)	372156 (28992)
Unique reflections	131079 (6434)	47163 (4594)	44772 (4755)	46487 (4297)	46654 (4550)	54536 (4437)
Multiplicity	6.8 (6.2)	6.8 (6.9)	6.7 (6.8)	6.8 (6.6)	6.8 (6.7)	6.8 (6.5)
Completeness (%)	99.9 (99.9)	99.5 (99.9)	97.6 (100)	99.9 (99.9)	99.9 (100)	100 (99.8)
Mean $I/\sigma(I)$	11.5 (3.7)	12.4 (4.9)	8.1 (3.6)	8.4 (3.3)	10.7 (3.8)	11.1 (4.2)
R_{merge} † (%)	11.5 (59.9)	13.8 (57.9)	17.6 (52.8)	15.7 (58.4)	12.5 (60.5)	13.5 (59.6)
R_{pim} †† (%)	4.7 (25.4)	5.7 (24.0)	7.3 (21.7)	6.5 (24.5)	5.2 (25.4)	5.6 (25.3)

Table 1: (**Continuation)**

Crystal data	AnβGal	AnβGal-E298Q /Allolactose	AnβGal-E298Q /3-Galactosyl-Glucose	AnβGal-E298Q /6-Galactosyl-Galactose	AnβGal-E298Q /4-Galactosyl-Lactose	AnβGal-E298Q /6-Galactosyl-Lactose
Molecules per ASU	1	1	1	1	1	1
Refinement						
R_{work} / R_{free} ††† (%)	15.02 / 17.14	16.24 / 20.04	14.97 / 19.32	17.86 / 23.20	16.88 / 20.41	19.08 / 22.74
Nº of atoms/average B ($Å^2$)						
Protein	7483 / 11.5	7483 / 21.1	7483 / 20.8	7483 / 23.0	7483 / 33.7	7483 / 28.1
Carbohydrate	372 / 24.9	367 / 39.9	334 / 37.5	389 / 43.2	400 / 57.4	400 / 44.8
Other	55 / 27.8	42 / 58.4	18 / 59.2	17 / 57.7		21 / 73.0
Water Molecules	1011 / 22.8	266 / 19.9	604 / 24.5	224 / 19.7	201 / 29.9	185 / 25.1
Ramachandran plot (%)						
Favoured	96.79	96.89	97.20	95.65	96.89	96.69
Outliers	0.10	0.10	0.00	0.10	0.00	0.00
RMS deviations						
Bonds (Å)	0.0061	0.0066	0.0074	0.0076	0.0086	0.0086
Angles (°)	1.2720	1.2105	1.3383	1.3104	1.4400	1.4078
PDB codes	5IFP	5IHR	5IFT	5JUV	5MGC	5MGD

$^{\dagger}R_{merge} = \sum_{hkl} \sum_i | I_i(hkl) - [I(hkl)]| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and [I(hkl)] is the weighted mean of all measurements.

$^{\dagger\dagger}R_{pim} = \sum_{hkl} [1/(N - 1)] 1/2 \sum_i | I_i(hkl) - [I(hkl)]| / \sum_{hkl} \sum_i I_i(hkl)$, where N is the redundancy for the hkl reflection.

$^{\dagger\dagger\dagger}R_{work} / R_{free} = \sum_{hkl} | Fo - Fc | / \sum_{hkl} | Fo |$, where Fc is the calculated and Fo is the observed structure factor amplitude of reflection hkl for the working / free (5%) set, respectively.

3. RESULTS

3.1. AnβGal overall structure

As previously reported (Rico-Diaz et al., 2014), we have purified and crystallized the β-galactosidase from *Aspergillus niger* (AnβGal). We present here its three-dimensional structure solved by molecular replacement at 1.71 Å resolution. Experimental and structure determination details are given in the Experimental Procedures section and in Table 1. The final model contains the mature protein without the residues of the signal peptide. The model consists of 967 residues with a molecular mass of 106 kDa as calculated from its primary structure. The chain extends from residue 41 to residue 1008, the last five histidines corresponding to the C-terminal affinity tag being not visible in the electron density map.

The glycosylation chains attached to the protein were mostly cleaved by Endo-H treatment applied prior to the crystallization step, which typically leaves primary N-acetylglucosamine (GlcNAc) units. However, several glycan chains of AnβGal were only partially cleaved, probably due to their poor access to the hydrolytic enzyme. From the electron density maps, GlcNAc units were modeled at positions Asn156, Asn373, Asn402, Asn478, Asn522, Asn622, Asn739, Asn760, Asn777 and Asn914, with glycosylation at sites Asn156, Asn478, Asn522 and Asn739 being not observed in other GH35 β-galactosidases. In agreement to that reported before for fungal GH35 β-galactosidases, extended glycan chains are attached to residues Asn373, Asn622 and Asn914 after the EndoH digestion.

The three-dimensional structures of three other GH35 β-galactosidases from fungus have been previously reported, those from *Penicillium* sp. (PsβGal; (Rojas et al., 2004), PDB code 1TG7), *Trichoderma reesei* (TrβGal; (Maksimainen et al., 2011), PDB code 3OG2) and *Aspergillus oryzae* (AoβGal; (Maksimainen et al., 2013), PDB code 4IUG). AnβGal shares highest identity with respect to AoβGal (75%) and PsβGal (73%) sequences, whereas 58% is found with respect to TrβGal sequence. Consequently, the AnβGal overall folding closely resembles that previously described for the other fungal enzymes, with a RMS deviations of only 0.7 Å after structural superimposition of the AnβGal coordinates onto the AoβGal and PsβGal structures (for 957 and 971 matched Cα atoms, respectively). The superimposition onto TrβGal structure gives a RMS deviation of 1.1 Å (on 986 matched Cα atoms).

As other fungal GH35 β-galactosidases, AnβGal is monomeric and folds into six domains (Figure 1A). It can be described as a central catalytic domain surrounded by five anti-parallel β-sandwich domains, arranged as a horseshoe or a letter C shape. The first domain is the catalytic and comprises residues Leu41 to Thr397, with a distorted $(\alpha/\beta)_8$ barrel topology in which the fifth helix is absent. This domain contains eight loops linking the β-α motifs (loops L1-L8), from which loops L3-L8 are exposed to solvent and configure the catalytic pocket that is located in the axis of the barrel (Figure 1, B-C). There are two disulfide bridges in this domain. The first one stabilizes loop β4-α4 (L4, residues Glu198-Asp212), and involves residues Cys205 and Cys206. Another disulfide bridge between Cys266 and Cys315 stabilizes loop β6-α6 (L6, residues Tyr260-Asn280) and is conserved in the three fungal enzymes. Moreover, there is a cis-peptide bond at

Tyr342-Met343, located at the end of strand β7, which is conserved among the reported GH35 β-galactosidases.

The second (Ala398-Val479), third (Ser480-Pro576) and fourth (Gln577-Pro665) domains have an anti-parallel β-sandwich structure that contains eight, nine and eight strands, respectively. These three domains, unique to the fungal β-galactosidases, are absent in bacterial and human GH35 β-galactosidases (Ohto et al., 2012). The fifth domain includes two different regions of the amino acid chain, Asp666-Leu683 and Tyr859-Tyr1006, and contains ten strands in an anti-parallel β-sandwich arrangement. Finally, the sixth domain, Pro684-Leu858, is inserted between both regions of the fifth domain, and also has an anti-parallel β-sandwich structure that contains eight strands. A long loop between strands sixth and seventh of this domain (residues 798-819, LP in Figure 1C) protrudes into the active site of the catalytic domain playing an important role in the enzymatic activity by making some of molecular contacts with the substrates, as will be explained below.

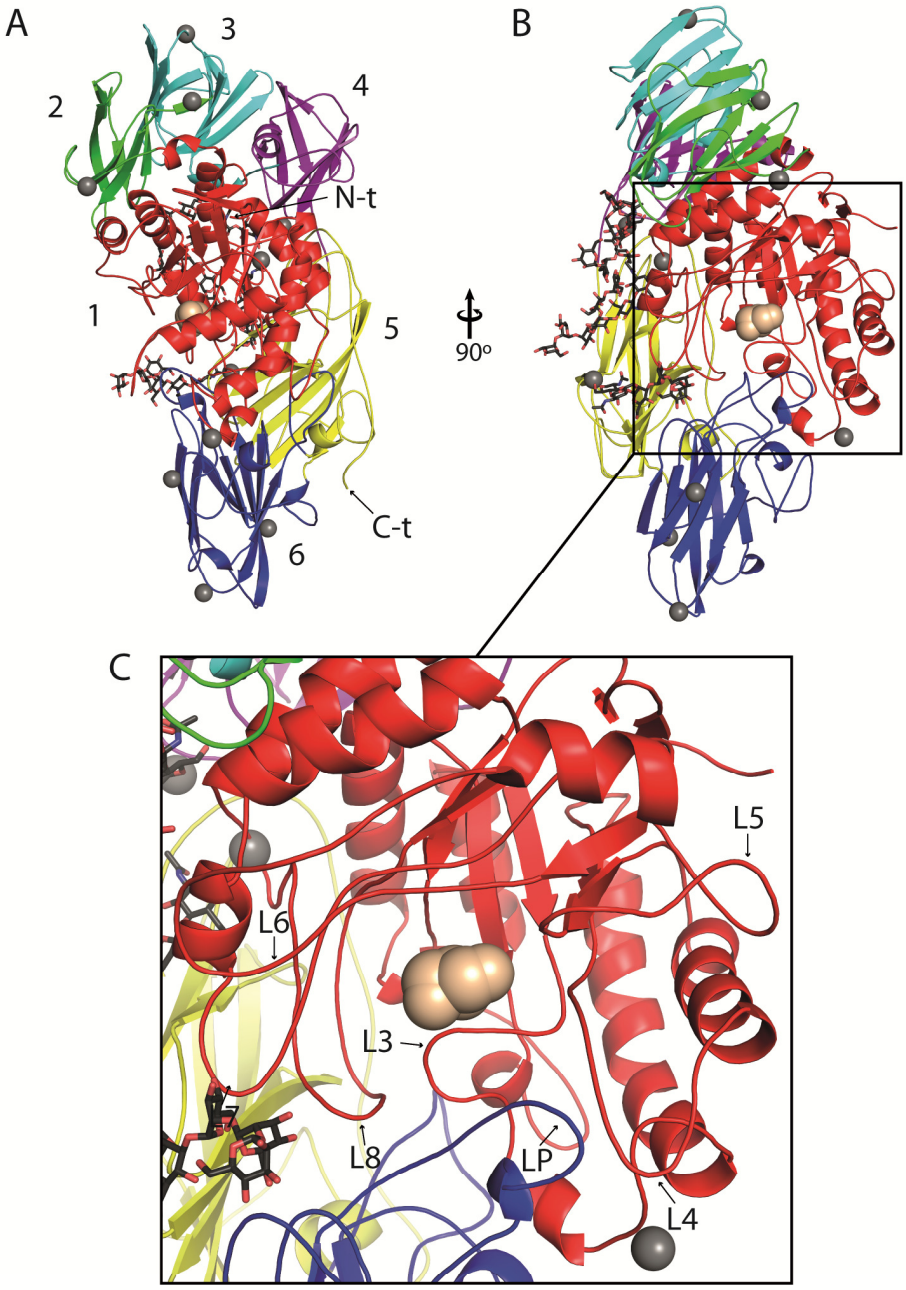
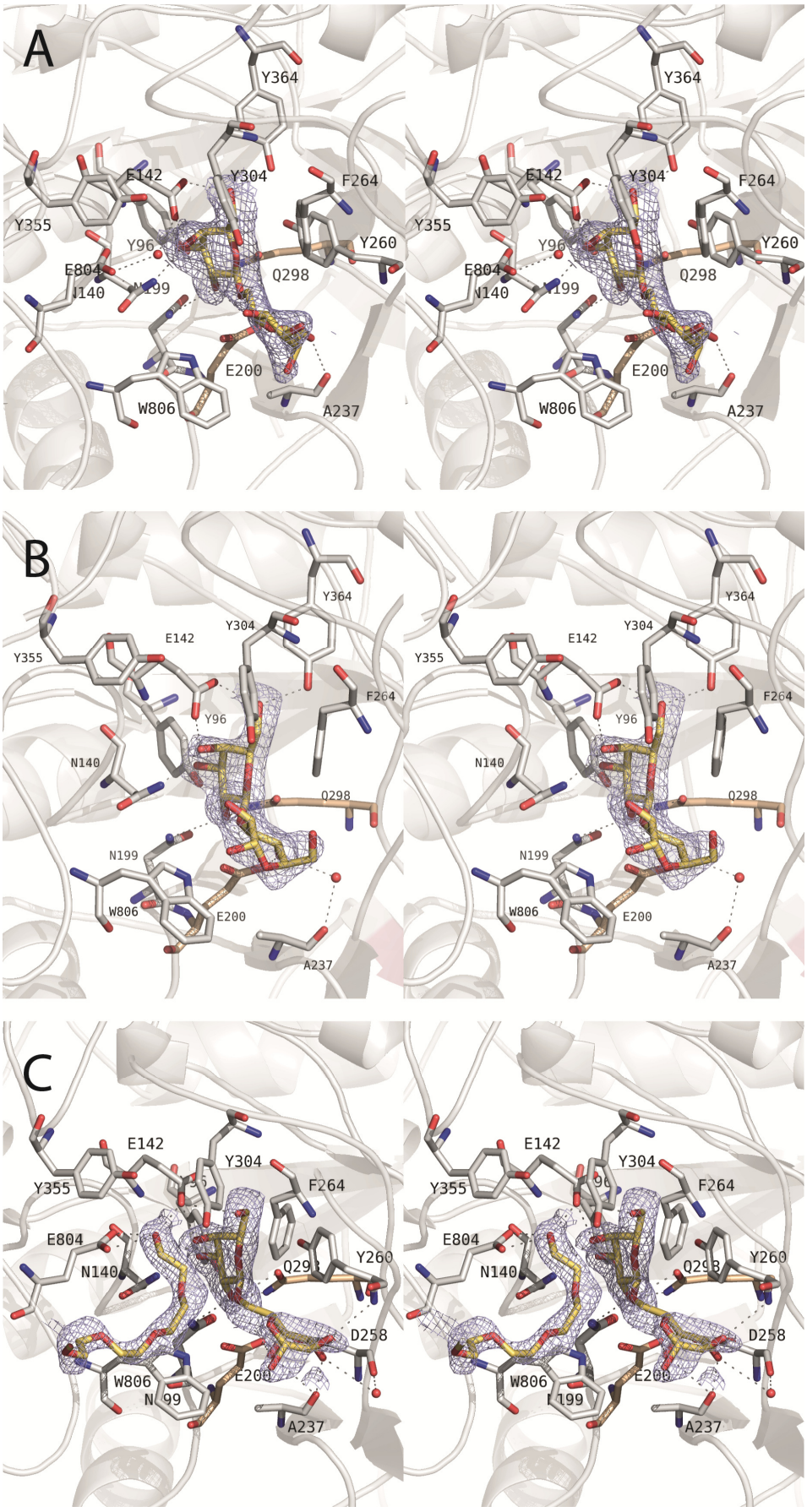


Figure 1: Structure of AnβGal. (A*)*, cartoon representation of AnβGal topology showing the catalytic domain (1) and the five β-sandwich domains (2-6). (B), 90º rotated view showing the active site. The frame (C) highlights the catalytic pocket, occupied by a glycerol molecule that is represented as beige spheres. The loops involved in substrate recognition are labeled. Glycosylation sites are represented as grey spheres and grey sticks represent long glycosylation chains at residues Asn373, Asn622 and Asn914.

A last interesting feature is loop L4 from the catalytic domain, which was not visible in the reported AoβGal model (Maksimainen et al., 2013). This loop is well ordered in the AnβGal crystals, where it is interacting with loop LP described above (Asp798-Leu812). Interestingly, the AnβGal loop L4 has a double substitution in residues Thr202 and Ser203, which are Ser and Gly in the other fungal β-galactosidases. Ser203 side chain makes an extra hydrogen bond with Thr807, reinforcing the interaction between both loops. Moreover, as we said above, this loop L4 has a disulfide bridge between Cys205 and Cys206, which was described in PsβGal (Rojas et al., 2004) but is not present in TrβGal (Maksimainen et al., 2011). This disulfide bridge could have a stabilization role in the loop structure.

3.2. Structure of the AnβGal Complexes

As said before, previous works have reported some complexes from other GH35 β-galactosidases with the product galactose, and also with non-hydrolysable sugar analogs, like IPTG, PETG or iminosugars like 1-deoxygalactonojirimycin (Maksimainen et al., 2011). However, a complex with oligosaccharides that may be natural substrates or products of GH35 β-galactosidases has not been described until now. Thus, in order to elucidate the different mechanisms and interactions involved in substrate recognition (hydrolysis) or in acceptor affinity (transgalactosylation), we solved the crystal structure of AnβGal in complex with different galacto-oligosaccharides, which may act as substrates or products depending on the catalytic reaction (hydrolysis or transgalactosylation) performed by the enzyme. These compounds are di- or trisaccharides, uncovering different glycoside bond-types between sugar units, including β(1-3), β(1-4) and/or β(1-6) linkages.


A
Y364
E142
Y304
F264
Y355
Y96
Y260
E804
Q298
N140
N199
E200
W806
A237
B
Y364
Y304
E142
Y355
F264
Y96
N140
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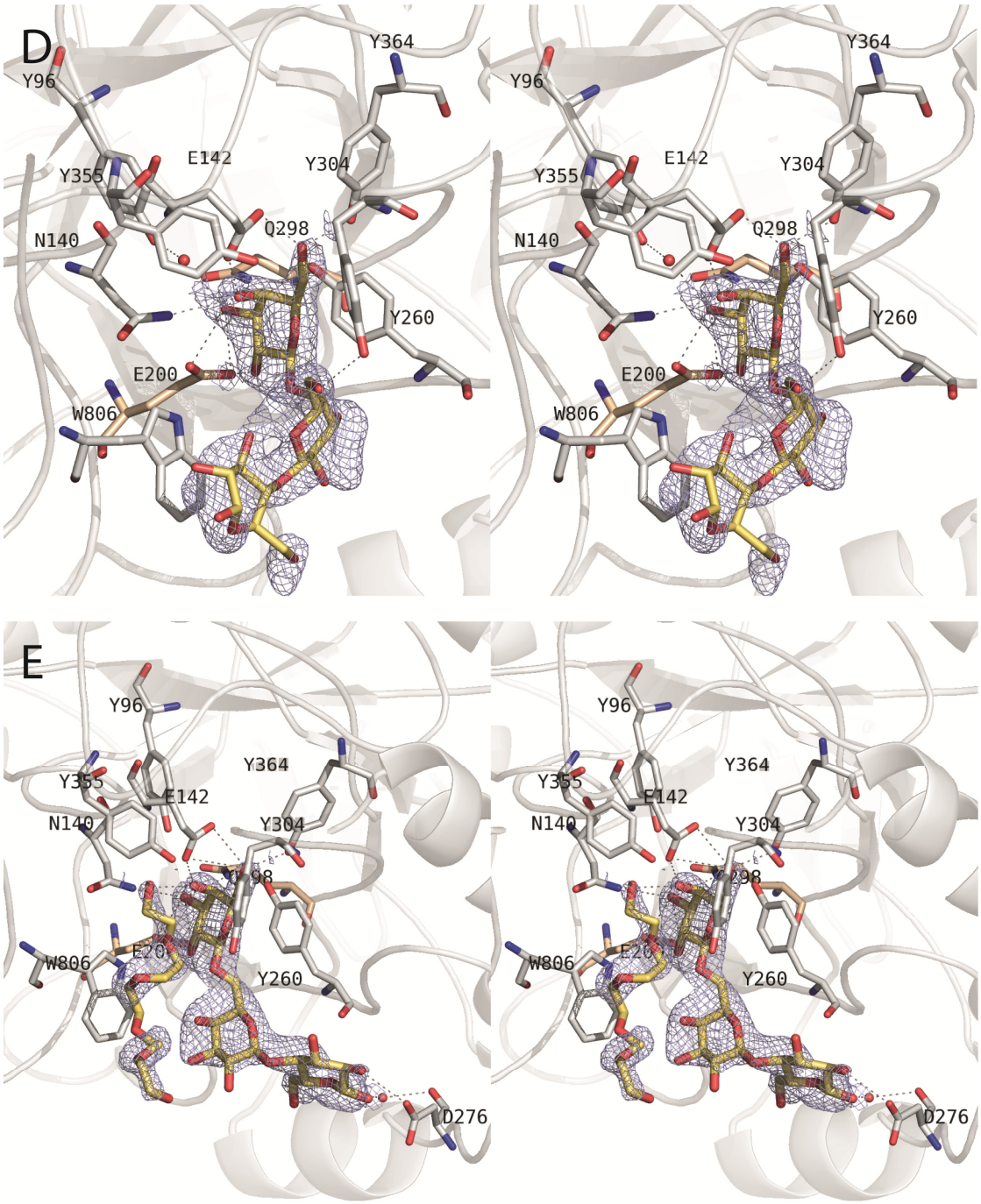

Figure 2: Structure of the E298Q AnβGal complexes. Stereo view of the active site, showing the relevant residues making interactions with each ligand. Main polar interactions are represented as dashed lines. The catalytic residues are highlighted in beige and the referred ligands in yellow. (A) Allolactose; (B) 3-Galactosyl-Glucose; (C) 6-Galactosyl-Galactose; (D) 4-Galactosyl-Lactose; (E) 6-Galactosyl-Lactose. A PEG molecule also bound at the active site is shown in (C) and (E). The final 2Fo-Fc electron density maps of the ligands, contoured at 0-9-1σ are shown in blue.

An inactivated E298Q mutant was generated to avoid the hydrolysis of the ligands, and crystallization trials were performed with the purified AnβGal-E298Q variant. Then, crystals were soaked into 6-galactosyl-glucose (allolactose, 6GalGlu), 6-galactosyl-galactose (galactobiose, 6GalGal), 3-galactosyl-glucose (3GalGlu), 4-galactosyl-lactose (4GalLac) and 6-galactosyl-lactose (6GalLac). The complexes with the disaccharides 6GalGlu, 6GalGal and 3GalGlu showed clear electron density in the active site defining their position (Figure 2). In the case of the 6GalGal complex, a molecule of PEG400 from the cryoprotectant solution is observed bound at the active site (Figure 2C). The complexes with the trisaccharides 4GalLac and 6GalLac showed clear electron density at the active center, but the ligands were apparently displaced from the -1 subsite, possibly due to some competition with the PEG3350 used as precipitant, which is visible in the complex with 6GalLac (Figure 2E). However, we consider these two complexes because its binding mode maintains most of the contacts in subsite -1 observed in the disaccharide complexes, and therefore could reveal important information of the interactions of larger substrates with residues from the catalytic pocket, and may define potential binding subsites.

In the complexes with the disaccharides (Figure 2, A-C), the non-reducing end of the sugars is placed at the galactose-binding pocket, defined as subsite -1, in a conserved way to that described previously in other complexes of the family (Cheng et al., 2012; Maksimainen et al., 2013; Maksimainen et al., 2011; Ohto et al., 2012; Rojas et al., 2004). Thus, the catalytic residues, Glu200 and Gln298 (Glu298 in the active native enzyme), fix galactose by making hydrogen bonds to its O2. Moreover, several other interactions stabilize galactose in a tight conformation, some

residues from loop L3 being mostly involved through both, main and side-chain atoms, by making hydrogen bonds with O3 (Ile139-O, Ala141-N), O4 (Glu142-OE2) and O6 (Glu142-OE2). Other residues as Asn199-ND2, Tyr96-OH and Tyr364-OH contribute tostabilize the galactose moiety by making hydrogen bonds with O2, O3 and O6, respectively.

Subsite +1 is mainly defined by hydrophobic interaction to Trp806 from loop LP but, interestingly, there are some differences in the binding mode of each substrate depending on the nature of the sugar unit attached to galactose and the β-linkage type. Thus, glucose in allolactose makes only one hydrogen bond to Ala237 main chain (O1…O) (Figure 2A), while that in 3GalGlu is stabilized through its O4 by a bifurcated hydrogen bond to Glu200 (O4…OE2) and to a water molecule that, in turn, is linked to Ala237 main-chain (Figure 2B). On the other hand, the second galactose in 6GalGal seems more tightly bound through hydrogen bonds to Tyr260 (O2…N) and Ala237 (O3…O and O4…O) main chains, and to Glu200 (O4…OE2) and a water molecule, which is linked to Asp258 (O) (Figure 2C). Moreover, the structural superposition of the complexes onto the native coordinates shows some changes in the position of Tyr304 and Phe264 side-chains depending on the molecule bound at the active center (Figure 3A). Therefore, these two residues, together with Trp806, make a hydrophobic platform that seems able to adapt to the different sugar rings.

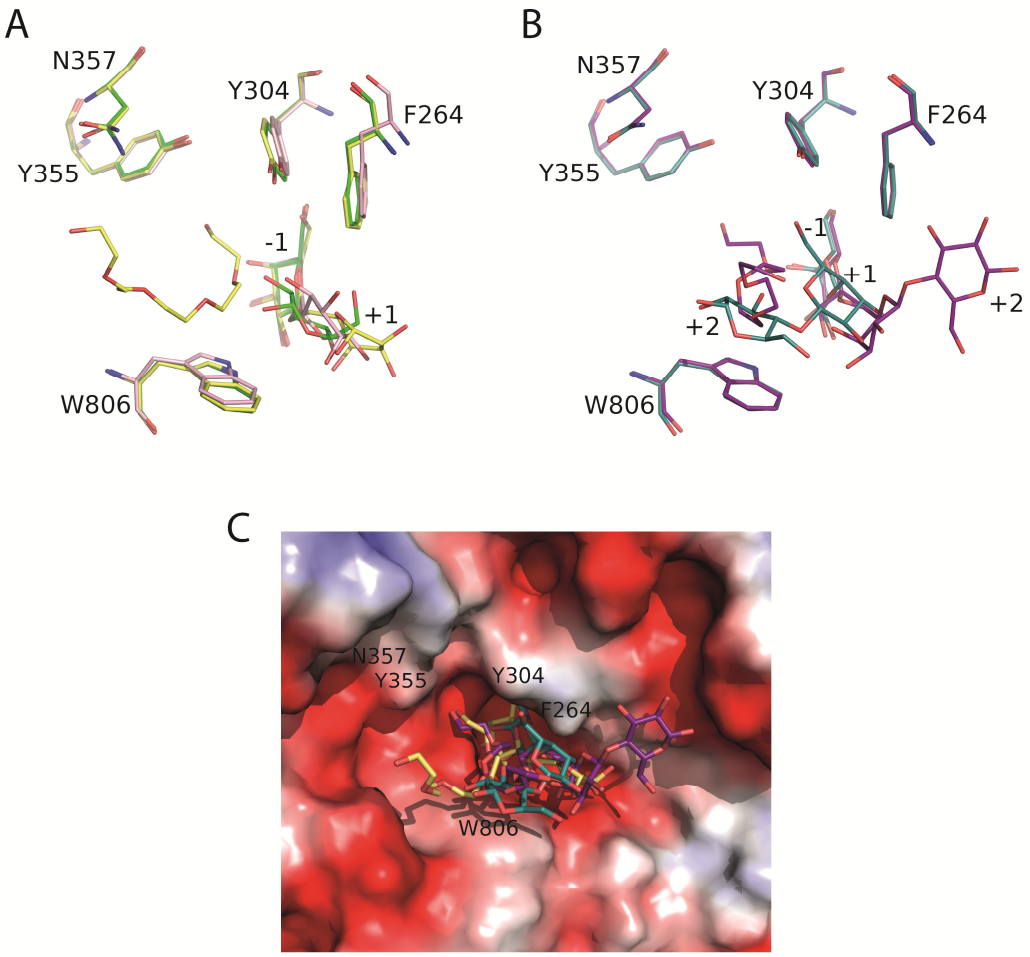

Figure 3: Conformations of residues and ligand binding modes in AnβGal active site. Superimposition of the complexes, showing different conformations depending on active site content: (A), the disaccharides Allolactose (*lightpink*), 3-Galactosyl-Glucose (*green*), 6-Galactosyl-Galactose (*yellow*) and (B), the trisaccharides 4-Galactosyl-Lactose (*cyan*) and 6-Galactosyl-Lactose (*purple*). (C), Stereo view of the molecular surface of AnβGal active site cleft showing the charge distribution (negative regions in red and positives in blue) and hydrophobic regions in white. Relevant residues for catalytic activity are labeled. Ligands are colored in yellow (6-Galactosyl-Galactose, PEG), cyan (4-Galactosyl-Lactose) and purple (6-Galactosyl-Lactose).

In summary, 6GalGlu, 3GalGlu and 6GalGal are placed in a similar way in the active site of AnβGal. However, while galactose at subsite -1 is stabilized by essentially the same interactions, there are some differences in the recognition pattern of the second ring of each ligand. Thus, all of them are accommodated by some polar interaction to loop L5, particularly with Ala237, but L6 is also involved in the 6GalGal complex binding, through residues Asp258 and Tyr260. Furthermore, residues Phe264 (loop L6), Tyr304 (loop L7), and Trp806 (loop LP) make a dynamic hydrophobic platform that stabilizes and accommodate the sugars at subsite +1.

As said before, the galactose bound at subsite -1 in the trisaccharide complexes is displaced from the canonical position observed in the other complexes. However, the galactose ring makes essentially the same interactions with residues of the active site, although in some cases through water molecules. The long chains of the residues involved in these contacts are flexible and, therefore, seem able to properly place galactose by only a slight movement making a productive complex (Figure 2, D-E). In the complexes reported here, the second galactose of 4GalLac and 6GalLac interacts weakly in subsite +1, and only one hydrogen bond is observed between its O6 and Tyr304 side-chain in the 4GalLac complex (Figure 2D).

The most interesting feature of these trisaccharide complexes is that their terminal glucose suggests two potential different +2 subsites. In the 4GalLac complex, glucose is stabilized by stacking with Trp806 side-chain, in a position similar to the PEG molecule found in the 6GalGal complex. In contrast, 6GalLac is oriented towards loop L6, its terminal glucose (O1) being stabilized by hydrogen bonds to Asp276 from loop L6 (Figure 2 D-E).

Consequently, the two trisaccharides display a different orientation that is possibly related to the AnβGal ability to accept a variety of substrates (Figure 3B). Likewise, the changes observed in residues Tyr304 and Phe264 at both trisaccharide complexes support their key role in recognizing the oligosaccharides, as proposed before for the disaccharides.

3.3. Mutation analysis of key aromatic residues within the active site

As said above, Trp806 seems crucial to accommodate the substrates in a precise orientation, while Phe264 and Tyr304 define a flexible hydrophobic patch that adjusts to the different substrate types upon binding. Phe264 and Trp806 are conserved among the β-galactosidases from yeast and fungus, while Tyr304 is unique to AnβGal, the other members having a Phe at this position. Thus, to confirm the important role of these residues on the catalytic function, a mutagenesis analysis of Tyr304 and Trp806 was performed.

As deduced from Table 2, the mutant Y304A losses more than 96% of the hydrolysis activity (Kcat/Km) against lactose, and this is mainly caused by a dramatically decline of the substrate affinity, as is shown by the elevated Km value of this mutant. The removal of the Tyr304 side-chain may affect the correct orientation of the adjacent Phe264, leading to a distortion deleterious to proper substrate binding. This effect is not produced by the mutation Y304F that, on the contrary, increases more than 33% the hydrolytic levels of WT, mainly thorough an increased Kcat. Therefore, the Tyr to Phe change probably enhances the formation of intermediate states along the mechanism pathway. On the other hand, the mutant W806S presents more than 90% loss of activity against lactose, while the more conservative mutation W806F still loses 43% of WT hydrolytic levels

through a higher binding affinity but a significant decreased catalytic efficiency. Thus, although lactose may have a stronger stacking interaction with Phe, the hydrolysis might be less efficient by incorrect substrate binding orientation.

Consequently, the suppression of the hydrophobic interactions at subsite +1, is accompanied by a dramatic decay of the hydrolytic activity of the enzyme. However, the nature of the aromatic residue at Trp806 position seems more crucial to maintain activity, supporting the critical role of this residue in the proper orientation of the substrate.

Table 2: Kinetic analysis of lactose hydrolysis by AnβGal variants and AoβGal. Kcat values were calculated assuming a protein molecular mass of 109 kDa. The ± sign refers to standard error curve fit using the kinetic module of Prism 6.

	WT	Y304F	Y304A	W806F	W806S	Y304F/Y355H/ N357G	Y304F/Y355H/ N357G/W806F	AoβGal
Kcat (s^{-1})	214.9±8.5	277.3±7.1	123.9±37.8	63.7±2.0	21.6±0.8	212.1±8.0	128.3±4.5	85.5±4.0
Km (mM)	92.5±7.4	89.5±4.6	1519±504.3	48.4±3.7	93.6±7.2	96.5±7.1	132.7±8.2	118.4±10.3
Kcat/Km	2.3	3.1	0.1	1.3	0.2	2.2	1.0	0.7

3.4. Mutation analysis of residues involved in transgalactosylation activity

Most fungal GH35 β-galactosidases are mainly used for the production of galacto-oligosaccharides in commercial applications, while AnβGal is known for its high hydrolytic activity. Comparison of the AnβGal active site to other GH35 β-galactosidases could give insights into the mechanisms that control the level of hydrolytic or transglycosylation activity of GH35 β-galactosidases.

An inspection to the solved structures of the family shows that most of the residues nearest to the catalytic Glu200 and Glu298 (Tyr96, Glu142, Asn199, Tyr364 and Trp806; see Figure 2) are well conserved in all eukaryotic GH35 enzymes. In addition, other residues as Ala237, Asp258 and Tyr260, all of them involved in making subsite +1 in the complexes here reported, are shared with other fungal GH35 β-galactosidases and, consequently, their role can be extrapolated to the other enzymes. However, there are a few residues unique to AnβGal, one of them being Tyr304 mentioned above. As explained before, this residue is shaping subsite +1, and is in close contact with Phe264. This not only suggests its potential role in the preference of AnβGal for hydrolysis, but also in the putative change in the donor substrate preference with respect to the other fungal GH35 β-galactosidases.

Moreover, the residues Tyr355 and Asn357 of AnβGal (Figure 3) are His and Gly in all the other fungal enzymes. Although these residues, located in loop β8-α8 (L8) are apparently distant from the catalytic pocket, they are shaping the wall of the channel giving access to the active site. In fact, together with Tyr304, they are contributing to accommodate the PEG molecule found in the crystals and, consequently, they may play a direct

role in substrate recognition. In relation to this, it is interesting to note that a superimposition of the complexes with different ligands shows changes also in the Asn357 side-chain (Figure 3A).

To determine the role of these residues in the hydrolysis/transglycosylation (H/T) ratio of AnβGal, we generated several mutant enzyme variants, by mimicking the residue composition of the other fungal GH35 β-galactosidases. For this purpose, and in addition to the active mutants described in the previous section (Y304F and W806F), a triple mutant Y304F/Y355H/N357G was obtained. Afterwards, GOS synthesis experiments were performed, including purified commercial Ao□Gal to investigate if the selected residues are responsible for the different catalytic features observed among all enzyme variants.

Analysis of maximum GOS yields (g of total tri- and tetra- galacto-oligosaccharides obtained during the synthesis per gram of initial lactose, in percentage) shows significant differences between WT and all the mutant enzymes tested (Figure 4). Thus, Y304F mutant reaches a maximum GOS yield of 20% (w/w), which is an improvement of 25% as compared to the maximum GOS yield of WT AnβGal. Also, mutant W806F and the triple mutant Y304F/Y355H/N357G show a maximum GOS yield of 23 and 22%, respectively (w/w). These values are 44% and 42% higher than the maximum GOS produced by the WT enzyme.

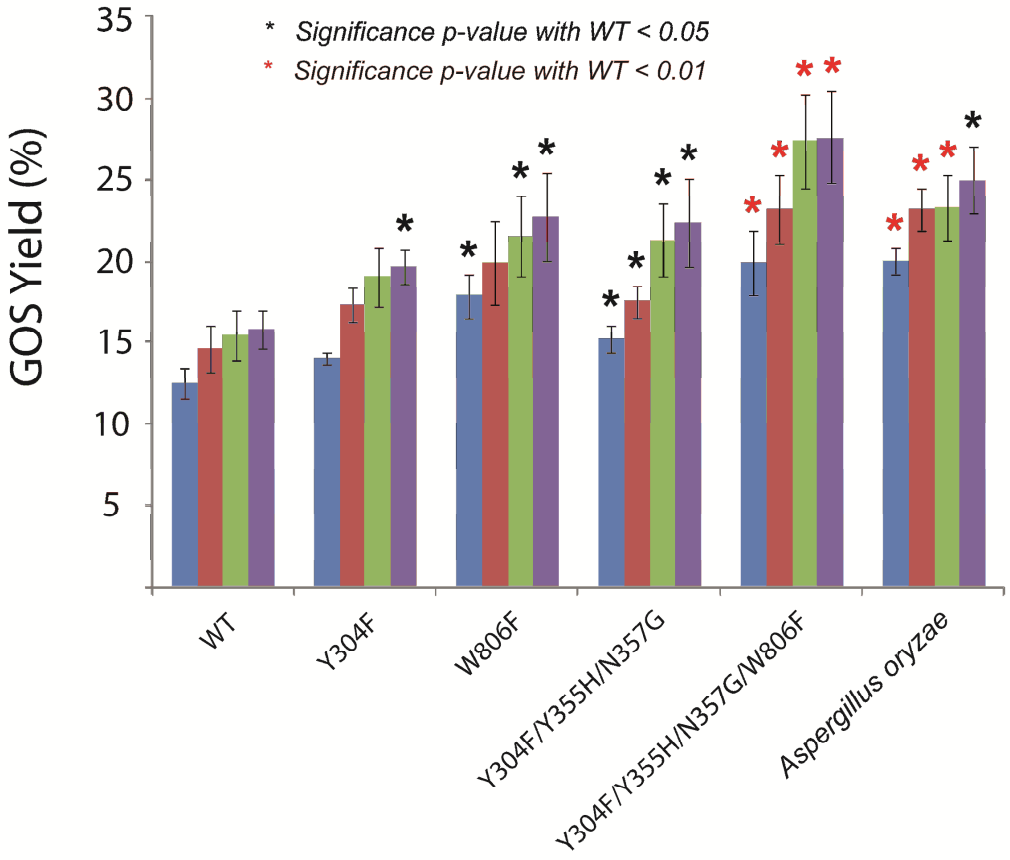

Figure 4: Galacto-oligosaccharides (GOS) produced over time and maximum GOS yield of different AnβGal variants. Yields represent the mass of total trisaccharides and tetrasaccharides GOS obtained during the synthesis per unit mass of initial lactose, in percentage. Three different replica of each were done and the values were averaged. GOS yield at: 3 hours (blue bars), 6 hours (red bars), 24 hours (green bars). Maximum GOS yield (purple bars). Asterisks indicate significant differences compared to wild type data.

To check if the improvements in GOS yields given by W806F variant and the triple mutant could be additive, a quadruple mutant Y304F/Y355H/N357G/W806F was obtained. The results show a great improve in maximum GOS yield, reaching values of more than 27% (w/w), which is 75% higher than the production observed in WT AnβGal. In fact, the quadruple mutant shows a higher level of GOS production than AoβGal (11% improvement between both maximum GOS yield averages), an

enzyme commonly used by industry because of its high transgalactosylation activity. In summary, the results showed an improvement of GOS synthesis levels in all the mutant variants tested, which therefore support the major role of the selected residues in catalytic properties of AnβGal.

We also performed kinetic measures of the different variants to identify in which way the diverse substitutions affect the hydrolytic activity. In most cases, an improvement in the GOS production involves a reduction in the hydrolytic activity of the enzyme (Table 2). Thus, as it happened with the W806F mutant, which losses 43% of hydrolytic efficiency (Kcat/Km) in comparison with WT AnβGal, the mutants Y304F/Y355H/N357G and Y304F/Y355H/N357G/W806F loss 5% and 58% respectively of the hydrolytic activity measured in WT AnβGal. The slight reduction in the triple mutant is likely due to the change Y304F that resulted in an important gain of hydrolytic levels, as we mention above, and this may counteract the loss of activity caused by the other two substitutions (Y355H and N357G).

4. DISCUSSION

Saprobic fungi like *A. niger* need to produce a large number of enzymes in order to break down plant biomass. One of these enzymes is AnβGal, which acts in conjunction with other carbohydrate-active enzymes to hydrolyze the intricate polysaccharide structures of plant cell wall. (Andersen et al., 2012; de Vries and Visser, 2001)

In this study, we described the structure of AnβGal and its complexes with some natural substrates. The native structure shares similar features with other GH35 β-galactosidases (Maksimainen et al., 2011; Maksimainen et al., 2013; Ohto et al., 2012; Rojas et al., 2004), arranging its polypeptide chain into six domains with a horseshoe disposition. A comparison between AnβGal and its complexes with the disaccharides 6GalGlu, 6GalGal and 3GalGlu and the trisaccharides 4GalLac and 6GalLac reveals the residues involved in each substrate-type recognition and supports the important role of three aromatic residues, Phe264, Tyr304 and Trp806. The differences in orientation of Phe264 and Tyr304 among complexes show a dynamic mechanism that allocates the +1 sugar depending on the structure of the ligand. This dynamic mechanism that involves residues Tyr304 and Phe264, combined with hydrophobic contacts to Trp806, could explain the plasticity against different hydrolytic substrates and transgalactosylation products of GH35 fungal β-galactosidases, which can cleave or make β(1-3), β(1-4) and β(1-6) galactosyl bonds. Although some conformational changes had been observed in the reported complex of TrβGal with artificial analogs like IPTG or PETG (Maksimainen et al., 2011), this is the first time that they are observed with natural substrates, which confirms the important role of these aromatic amino acids in the catalytic

performance of the enzyme. However, our complexes do not show the switch at residues Trp806 and Glu200 upon ligand binding, described in the TrβGal complexes. Therefore, the changes in Trp806 and Glu200 orientation might be an artifact due to the IPTG and PETG binding, as the authors suggested in their work. To confirm the implications of the residues making this hydrophobic platform in catalytic activity, mutagenesis analysis were performed. Kinetic studies of AnβGal variants with mutations in positions Tyr304 and Trp806 support the importance of the hydrophobic contacts made by these amino acids with the substrate in the proper hydrolytic function of the enzyme.

An interesting finding in the soaking experiments of AnβGal with the trisaccharides 4GalLac and 6GalLac shows that the sugar chains might be oriented in the active site in two different ways depending on the structural features of the oligosaccharide. In nature, AnβGal acts cooperatively with other *A. niger* enzymes to hydrolyze components of plant material. These components, like hemicelluloses or pectin, are very complex in composition and their hydrolysis generates a mix of polysaccharides that can be partially ramified. It has been reported that β-galactosidase activity is required for degradation of complex polysaccharides containing terminal galactose units, like galactoglucomannan, xylan, xyloglucan and arabinogalactan type I, by *A. niger* (Andersen et al., 2012). In this context, the broad cavity housing the AnβGal active site, which may allocate the trisaccharides in two different orientations, exhibits visible grooves at its molecular surface that might also permits the anchoring and hydrolysis of these branched substrates (see Figure 3B). Therefore, the peculiar shape of AnβGal active site can

confer the enzyme an advantage for the efficient processing of the complex net of polysaccharides composing the plant material.

Apart from the hydrolytic biological function of AnβGal in degrading cellular walls components, its ability to catalyze transgalactosylation makes it interesting for being used in the synthesis of prebiotics like GOS in industrial applications. We generated improved mutant variants after selecting potential key residues by the structural comparison of AnβGal to other GH35 fungal β-galactosidases with different catalytic features. The reduced hydrolysis observed in variants with more transgalactosylation ability is a common consequence of the change in acceptor preference that modifies the hydrolysis/transglycosylation (H/T) ratio (Feng et al., 2005; Kim et al., 2008; Placier et al., 2009; Rivera et al., 2003). Several features have been described in retaining glycoside hydrolases that could modulate the acceptor preference of the enzyme. In turn, this acceptor preference will determine a preferred hydrolytic activity (water as acceptor) *vs.* a significant transglycosylation activity (sugar moieties as acceptors) (Bissaro et al., 2015). For instance, in some glycoside hydrolases like neopullulanases, endo-β-N-acetylglucosaminidases or maltogenic amilases, the creation of a more hydrophobic environment by mutagenesis promotes the binding of sugar moiety and disturbs the access to the incoming water molecule thus modifying the hydrolysis/transglycosylation ratio (Abdul Manas et al., 2015; Yin et al., 2009)

Interestingly, the analysis of the catalytic pocket always results in the presence of more hydrophilic residues in AnβGal, as compared to the other fungal GH35 β-galactosidases. Thus, mutants obtained from AnβGal mimic the hydrophobic environment found in other fungal β-

galactosidases of the family, resulting in a higher tendency of sugars *vs.* water to be acceptors and, consequently, in an increased transgalactosylation ability. Furthermore, the additional substitution of Trp806 to Phe, a more hydrophobic amino acid, also results in an increase of the transgalactosylation activity of the enzyme, supporting this hypothesis.

The results give insight into the features that define the nature of the enzymatic activity in β-galactosidases of GH35 family. Moreover, the ability of getting mutants with different levels of hydrolytic or transgalactosylation activity offers new valuable options for the biotechnological industry, depending on the application goals. Furthermore, the similarities among active sites of GH35 fungal β-galactosidases, open the door to the improvement of other enzymes by analogous modifications.

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Chapter 3

Rational mutagenesis by engineering disulphide bonds improves *Kluyveromyces lactis* β-galactosidase for high-temperature industrial application

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ABSTRACT

Kluyveromyces lactis β-galactosidase (KlβGal) is one of the most important enzymes in the dairy industry. The poor stability of this enzyme limits its use in the synthesis of galactooligosaccharides (GOS) and other applications requiring high operational temperature. To obtain thermoresistant variants, a rational mutagenesis strategy by introducing disulfide bonds in the interface between the enzyme subunits was used. Two improved mutants, R116C/T270C and R116C/T270C/G818C, had increased half-lives at 45ºC compared to KlβGal (2.2 and 6.8 fold increases, respectively). Likewise, Tm values of R116C/T270C and R116C/T270C/G818C were 2.4 and 8.5ºC, respectively, higher than KlβGal Tm. Enrichment in enzymatically active oligomeric forms in these mutant variants also increased their catalytic efficiency, due to the reinforcement of the interface contacts. In this way, using an artificial substrate (*p*-nitrophenyl-β-D-galactopyranoside), the Vmax values of the mutants were ~1.4 (R116C/T270C) and 2 (R116C/T270C/G818C) fold higher than that of native KlβGal. Using lactose, the Vmax for R116C/T270C/G818C almost doubled the Vmax for KlβGal. Validation of these mutant variants of the enzyme for their use in applications that depend on prolonged incubations at high temperatures was achieved at the laboratory scale by monitoring their catalytic activity in GOS synthesis.

1. INTRODUCTION

β-D-galactosidases (EC 3.2.1.23) are enzymes that catalyze the hydrolysis of terminal non-reducing β-D-galactose units from β-D-galactosides. They have been mainly used for the hydrolysis of lactose in milk and other dairy products. β-galactosidases also have transgalactosylation activity that make them very attractive for obtaining galactooligosaccharides (GOS) (Panesar et al., 2006). GOS are prebiotic milk derivatives, which nowadays are included in some foods (Sangwan et al., 2011) and might to be beneficial to consumer's health, such as prevention of colorectal cancer (Bruno-Barcena and Azcarate-Peril, 2015), avoidance and treatment of symptoms in asthmatic disease (Verheijden et al., 2015), or improvement in the microbiota and certain markers of immune function in elderly people (Vulevic et al., 2015). The convenience of adding GOS to infant formula has also been proposed recently, since there seems to be some beneficial effects on gut microbiota, metabolic activity, stool consistency and frequency, and the amelioration of certain immune markers in babies (Vandenplas et al., 2015). The biggest drawback of the enzymatic production of GOS comes from the need for working at high lactose concentration, since transgalactosylation is favored under these conditions over other enzymatic activities. The poor solubility in water of this disaccharide means that transgalactosylation reactions must run at relatively high temperatures to reach the required lactose concentration in solution (Gosling et al., 2010).

β-D-Galactosidase from the yeast *Kluyveromyces lactis* (KlβGal) is widely used in the food industry. However, the stability of native KlβGal (Jurado et al., 2004) limits its catalytic activity in applications that need high

temperatures, e.g. the production of GOS (Akiyama et al., 2001). Even though thermophilic bacteria might offer more versatility for obtaining robust enzymes for this technology, the confirmed GRAS (Generally Recognized As Safe) condition of yeasts, like *K. lactis* and *K. marxianus*, and fungi, like *Aspergillus niger* and *A. oryzae*, places them among the favourite sources (Rubio-Texeira, 2006). Some approaches use KlβGal to improve its thermal stability; these include immobilization using functionalized multi-walled carbon nanotubes (Ansari et al., 2013), quitosan particles (Klein et al., 2013) and polystyrene nanofibers (Misson et al., 2016).

Protein engineering is widely used to increase enzyme stability (Iyer and Ananthanarayan, 2008) by using directed evolution methods (error prone PCR and DNA shuffling), semi-rational methods such as CASTing, or rational methods supported by the study of the structure of the enzymes (Dalby, 2011; Hibbert and Dalby, 2005; Yang et al., 2014). However, there are currently no published reports on protein engineering strategies for improving the thermal stability of KlβGal.

From our structural studies (Pereira-Rodríguez et al., 2012), we found that KlβGal (PDB code 3OBA) is a tetrameric enzyme with an oligomerization pattern of “dimerization of dimers”, with higher dissociation energy for the dimers than for the tetramer. Based on this knowledge and in depth analysis of its catalytic sites (Pereira-Rodríguez et al., 2012), we hypothesized that design strategies used in other systems (Fernandez-Lafuente, 2009) based in the substitution of some target residues in the contact interfaces between dimers, thus enabling new stabilizing interactions (Fernandez-Lafuente, 2009), could effectively increase the thermal stability of the enzyme.

We present here work based on this rational strategy for improvement of the thermoresistance of the KlβGal enzyme. The catalytic and stability properties of 2 active variants obtained by introducing disulfide bonds between the oligomers are compared with the native enzyme. Their potential value in industrial applications needing high temperatures has been validated by measuring their transgalactosylation activity in GOS production.

2. MATERIAL AND METHODS

2.1. Gene cloning and protein purification

The *LAC4* gene (Gene ID 2897170) was amplified by PCR from the pLX8 plasmid and cloned in the YEpFLAG vector (*Eastman Kodak Company*) as previously reported (Becerra et al., 2001a). Mutagenesis of KlβGal was done by PCR with the commercial kit Quikchange-XL (*Stratagene*), oligonucleotide design and mutagenesis following the manufacturer's recommendations. The construction was used to transform *Saccharomyces cerevisiae* BJ3505 cells (*Eastman Kodak Company*) with the commercial kit *Frozen-EZ Yeast Transformation Kit II™* (*Zymo Research)*.

For protein extraction and purification, cells were grown at 30ºC and 250 rpm for 72 h in a 2 L Erlenmeyer flask containing 1 L YPHSM medium [1% (w/v) glucose, 3% (v/v) glycerol, 1% (w/v) yeast extract and 8% (w/v) peptone]. Under these conditions, there was increased protein expression.

Protein extracts were obtained by mechanical procedures from pelleted cells as previously described (Becerra et al., 2001b).

Proteins were purified using *ANTI-FLAG M2* affinity Gel (*Sigma*) packed in 10 mL chromatography columns (Bio-Rad 731-1550). A column with 0.3 mL of affinity gel was equilibrated with TBS (150 mM NaCl, 50 mM Tris-HCl pH

7.4). Elution of the bound FLAG fusion protein was made possible by competition with a solution containing 150 µg/mL FLAG peptide (*Sigma*).

2.2. Protein model analyses

Preselection of the cysteine target residues was done with SSBOND software (Hazes and Dijkstra, 1988). Analysis of interfacial surfaces required the Protein Interfaces, Surfaces and Assemblies Service (PISA) at the European Bioinformatics Institute (Krissinel and Henrick, 2007). Analyses of protein structures, target selection for mutagenesis and result analyses involved the software programs PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) and Coot (Emsley and Cowtan, 2004). In most cases**,** to predict the stability of a mutated structure was used I-Mutant 2.0. software (Capriotti et al., 2005).

2.3. β-PNPG hydrolytic activity measurement and kinetics

Enzymatic activity was measured using *p*-nitrophenyl-β-D-galactopyranoside (β-PNPG). Cellular protein extracts or purified protein preparations were diluted in 150 µL Z buffer (100 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.6 mM $MgSO_4$). After incubation for 4 min at 30ºC, the reaction was started by adding 150 µL substrate in Z buffer to the enzyme solution. Aliquots (100 µL) of the reaction mixture were stopped at 2 time-points by adding 100 µL 1 M Na_2CO_3. Released *p*-nitrophenol was measured by UV absorbance at 400 nm. β-Galactosidase activity is expressed in enzymatic units (U) defined as the amount of enzyme capable of releasing one µmol of product (*p*-nitrophenyl) per min (i.e. µmol min^{-1} mL^{-1}) under the experimental conditions.

Kinetic characterization of KlβGal and mutants were based on the assaying of β-galactosidase activity of purified protein samples (as described above) at different substrate concentrations (0-20 mM). Measurements were made in triplicate with 0.3 μg mL^{-1} enzyme. Non-linear fitting was based on least-squares to infer the apparent enzymatic kinetic parameters from Michaelis-Menten plots, using Prism 6.00 for Windows (*GraphPad Software Inc.*).

2.4. Kinetics of lactose hydrolysis

Kinetics of lactose hydrolysis were measured by the glucose produced by the enzyme at different lactose concentrations. Purified samples were diluted in Z buffer. The initial velocity was measured in triplicate with 5.5 μg mL^{-1} enzyme and lactose from 0 to 160 mM. The reaction times were 6-20 min at 30ºC. The reaction was stopped by heating to 96ºC for 5 min.

β-galactosidase activity is expressed in enzymatic units (U), defined as the amount of enzyme capable of liberating 1 μmol of product (D-glucose) per min under the experimental conditions (i.e. μmol min^{-1} mL^{-1}).

Glucose concentration was measured using the commercial kit D-Glucose GOD-POD (Nzytech). Non-linear fitting by the least squares method was used to infer the apparent enzymatic kinetic parameters from Michaelis-Menten plots (via Prism 6).

2.5. Determination of disulfide contacts

To examine the formation of disulfide bonds in KlβGal and its mutants, mass spectrometry and colorimetric assays were done.

Two different mass spectrometry analyses were performed, Nano-scale LC-MALDI-MS and Nano-LC-QTRAP 5500, as described previously (Fernández-

Puente et al., 2017; Lourido et al., 2016). Identification of proteins was performed using the Protein Pilot software 4.5 (ABSciex). Search parameters were set with trypsin cleavage specificity, iodoacetamide (IAA) modified cysteine as fixed modifications (when required), biological modification "ID focus" settings, and a protein minimum confidence score of 95% (Detected Protein Threshold >95%, Unused ProtScore >1.3).

DTNB [5,5´-dithiobis(2-nitrobenzoic acid)] colorimetric assay (Ellman, 1959) was also conducted. Protein samples were dissolved in TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.4) with guanidine hydrochloride 3mM and divided in two aliquots. One of the aliquots was treated with Pierce™ Immobilized TCEP Disulfide Reducing Gel (*ThermoFisher Scientific*) for an hour to reduce disulfide bonds. Samples (non-reduced and reduced) and DTNB solution (2mM) were mixed in a 1:1 proportion. After incubation at 37ºC for 10 minutes, absorbance at 412 nm of mixtures was measured. DTT was used to make the standard curve. Measurements were made in triplicate. Statistical significant differences ($p>0.05$ and $p>0.01$) of sulfhydryl groups concentration between non-reduced and reduced conditions of each variant were tested by using a two-tailed Student´s test.

2.6. Thermostability analyses

Thermal stabilities of protein variants (mutants and native enzyme) were determined by 2 procedures. In the first, thermostability was determined by measuring the residual activity of variants after incubation. Protein samples were incubated in Z buffer for different times at 42 and 45 ºC.

Differential Scanning Fluorometry (DSF) was used to obtain the melting temperature of both variants (Ericsson et al., 2006; Niesen et al., 2007).

The Reaction mix was composed of pure protein (4 µM) and Sypro Orange dye (*Sigma*) at 10x final concentration in a total volume of 25 µL. We used 96-well thin-wall PCR plates (*Thermo*) sealed with *Optical-Quality Sealing Tcape* (BioRad). Samples were incubated for 5 min at 15ºC and heated from 15º to 90°C with a ramp rate of 0.5°C min^{-1} in a real-time PCR machine (*iCyclerIQ, BioRad*).

Fluorescence of the dye was continuously monitored. The excitation and emission wavelengths were 490 and 530 nm, respectively. Fluorescence intensity was plotted as a function of temperature and a non-linear Boltzmann fit used the Prism 6 program. The melting temperature was defined as the temperature corresponding to the peak of the first derivative of this curve (Niesen et al., 2007; Niesen et al., 2007). Media from 3 independent experiments were obtained.

2.7. Sedimentation velocity assays (SV)

Samples (320 µL) in 50 mM Tris-HCl and 150 mM NaCl at pH 7.4 were loaded intoanalytical ultracentrifugation cells. Two different enzyme concentrations were used, 0.1 and 0.2 mg/mL. The experiments were carried out at 48 k rpm in a XL-I analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with UV-VIS absorbance and Raleigh interference detection. Sedimentation profiles were recorded at 275 nm. Sedimentation coefficient distributions were calculated by least-squares boundary modelling of sedimentation velocity data using the continuous distribution c(s) Lamm equation model (implemented by SEDFIT 14.7g) (Schuck, 2000). Experimental *s* values were corrected to standard conditions (water, 20°C, and infinite dilution) using the program SEDNTERP (Laue et al., 1992) to get the corresponding standard *s* values ($s_{20,w}$).

2.8. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide Gel Electrophoresis used previously reported procedures (Becerra et al., 1997).

In the case of the native PAGE, the use of sodium dodecyl sulfate (SDS) in gel and buffers was avoided, and electrophoresis ran at 4ºC. In all cases, polyacrylamide gels were stained with *Coomasie Brilliant Blue* (Westermeier, 2006).

2.9. Galactooligosaccharide measurement

GOS and lactose concentrations were determined by HPLC (HPLC Waters Breeze I), using a Waters Sugar-Pak column eluted at 90 ºC with Milli-Q water at a flow rate of 0.5 mL/min, and a Waters 2414 refractive-index detector.

Reactions involved mixing 0.0006 U (U as defined in lactose kinetics) of pure protein in phosphate buffer 0.1 M (pH 6.8), supplemented with 40% lactose. Samples (500 µL) were incubated at different temperatures and 300 rpm. Samples were taken at 0, 2, 4, 6, 8, 24 and 48 h. Reactions ran at 40ºC.

Carbohydrates were quantified by external calibration, using standard solutions of galactose, glucose, lactose, raffinose and stachyose.

3. RESULTS AND DISCUSSION

3.1. Design of a stabilization strategy for KlβGal

KlβGal adopts 2 active oligomeric forms in solution, as a dimer or a tetramer formed by a dimer of dimers (Pereira-Rodríguez et al., 2012). The predicted dissociation energy of the dimers in the tetramer is lower than that of the monomers in the dimer. The structure of the combined

monomers (4 identical subunits A, B, C, D) in the tetramer and dimers is shown in Figure 1. Although not a general rule, there is evidence supporting the notion that, in families of multimeric enzymes, members with the highest thermal stabilities also have the highest oligomerization states (Clantin et al., 2001; Maeda et al., 2002; Walden et al., 2001). Reduction in the accessible surface area produced by the joining of the subunits has also been associated with thermostability (Jaenicke and Böhm, 1998). As a result of this rational strategy, different proteins (e.g. malate dehydrogenase and cocaine esterase) have been successfully engineered to form and stabilize oligomers (Bjørk et al., 2003; Fang et al., 2014).

KlβGal has detectable enzymatic activity only in its dimeric and tetrameric forms (Becerra et al., 1998), which can be attributed to modifications at the entrance to the catalytic pocket after dimerization of the monomers (Pereira-Rodríguez et al., 2012). Therefore, our stabilization strategy has been focused on the reinforcement of KlβGal quaternary structure. To strengthen the interface contacts between monomers and between both dimers, a prediction of putative new inter-molecular interactions was made by investigating the interface areas of the oligomer using 2 molecular visualization softwares, Pymol and Coot.

Along with these considerations derived from studies of oligomer interface surfaces using several molecular graphic softwares described in Methods, the selection of target residues also took into account features such as B-factor values and stability changes produced by the newly introduced amino acid residues. Residues with the highest average B-factors correspond to those with the highest thermal motion and flexibility of a protein (Reetz et al., 2006) . Inversely, there is a direct relationship

between the degree of rigidity and protein thermostability (Jaenicke and Böhm, 1998). The properties of the modified molecular interactions due to these substitutions, the residue distances, dihedral angles and energetic constrains were also calculated.

Three mutation targets (pairs Val2-His817, Gly37-Ser260 and Gly818-Cys3) were selected from the monomer-monomer contact surfaces of the dimers (between subunits A-B or C-D, Figure 1B) and 4 (pairs Asp116-Thr270, His425-Tyr872, Leu601-Glu922 and Gly983-Gly983) from the main dimer-dimer interface (between subunits A and B, Figure 1A; Table 1). In all cases, the selected residues were mutated to cysteines to get new disulfide bonds due to the covalent nature of these molecular interactions, which could maximize reinforcement of the interface surfaces.

Therefore, to insert disulfide bonds at the monomer-monomer interface, mutants V2C/H817C, G37C/S260C and G818C were obtained. However, the mutants R116/T270, H425C/T872C, L601C/E922C and G983C were generated to form disulfide bridges at the dimer-dimer interface.

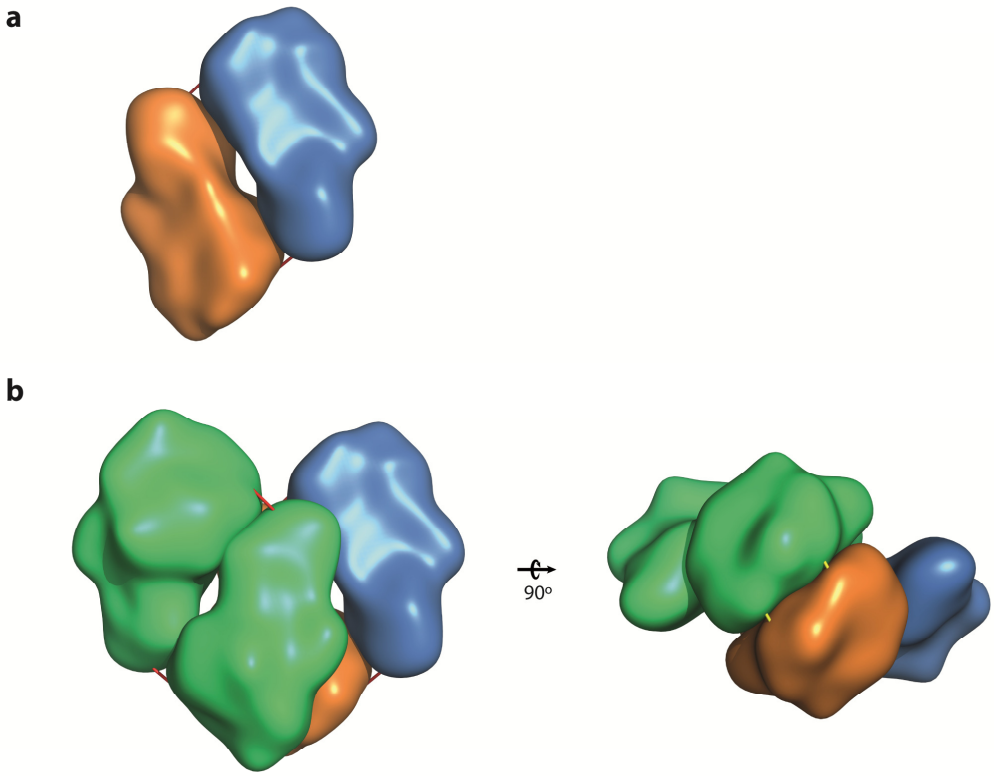

Figure 1: Surface representation of KlβGal topology showing the oligomeric organization. A, Dimer made up by subunits A (orange) and C (blue). Red bars represent the location of disulfide bonds between Cys3 (native) and Cys818 in mutant R116C/T270C/G818C. B, tetramer made up by dimer of subunits B and D (green), and dimer of subunits A (orange) and C (blue). Red bars represent the location of disulfide bonds between Cys3 (native) and Cys818 in mutant R116C/T270C/G818C and yellow bars represent the location of disulfide bonds between Cys116 and Cys270 in mutants R116C/T270C and R116C/T270C/G818C.

3.2. Screening and verification of mutants

Protein extracts from cells carrying these variants were analyzed by measuring thermal stability and residual catalytic activity, using β-PNPG as the substrate. Thermal stabilities were tested after 20 min incubation at 42.5ºC, as compared to the residual activity of the native enzyme given the same treatment. In 3 cases (G37C/S260C, G818C and G983C), there was no

increase in their residual activity compared with the natural enzyme. Furthermore, the residual activity of 2 mutants (V2C/H817C and L601C/E922C) was 10% lower, and H425C/T872C had no enzymatic activity (Table 1). This undesirable feature was interpreted as a consequence of the breakage of other important intermolecular linkages between the subunits, e.g. hydrogen bonds.

Table 1: Main properties of KlβGal variants obtained.

Mutant	Subunit Interface involved	Residual activity * change compared with native	Presence of hydrolytic activity (P=presence/NP=not presence)
V2C/H817C	Monomer-Monomer	<10%	P
G37C/S260C	Monomer-monomer	=	P (<20%)
G818C	Monomer-monomer	=	P
R116/T270	Dimer-dimer	>10%	P
H425C/T872C	Dimer-dimer	-	NP
L601C/E922C	Dimer-Dimer	<10%	P
G983C	Dimer-dimer	=	P
R116C/T270C/G818C	Monomer-Monomer Dimer-Dimer	> 25%	P

*Residual activity: Remaining activity after 20 minutes incubation at 42.5ºC

Interestingly, R116C/T270C which has residue changes located in the dimer-dimer interface, had a noticeable increase (>10%) in the residual

activity (Table 1). Analysis of this variant by SDS-PAGE, with and without addition of β-mercaptoethanol used to break disulfide bonds, showed the presence of larger complexes of this mutant enzyme under oxidizing conditions, thus suggesting the existence of disulfide bonds between both dimers (Figure 2). Because a pair of mutated oligomers contributes to the dimer-dimer interface, 2 disulfide bonds are formed that further strengthen the final structure of the tetramer (Figure 1B).

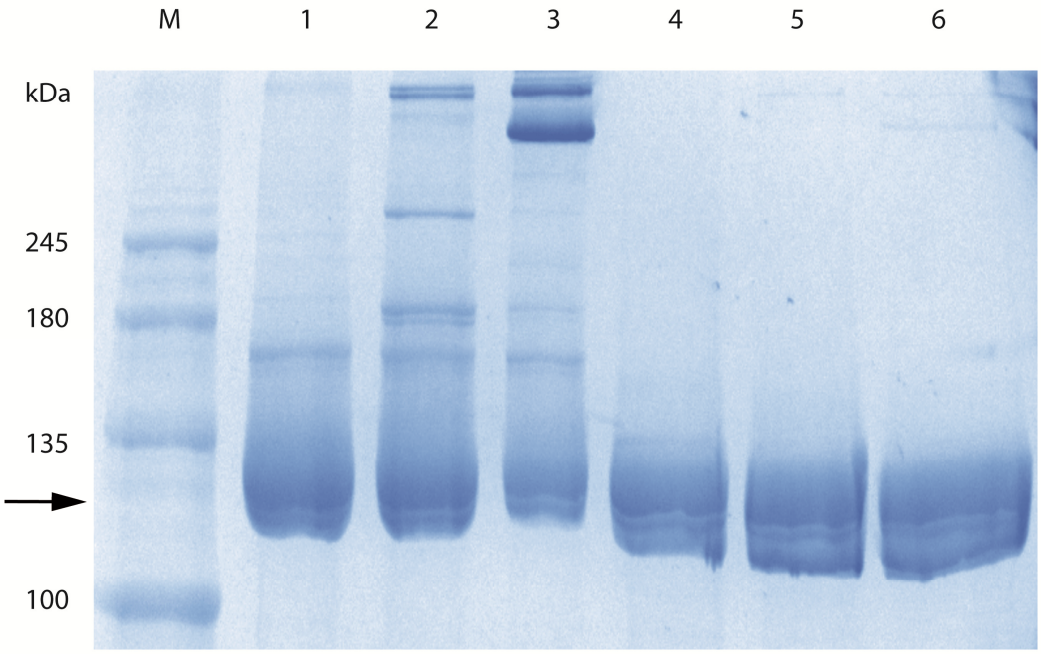

Figure 2: SDS PAGE of KlβGal and its mutants in oxidizing and reducing conditions. M, protein MW marker; 1, KlβGal without 2-mercaptoehtanol; 2, R116C/T270C without 2-mercaptoehtanol; 3, R116C/T270C/G818C without 2-mercaptoehtanol; 4, KlβGal with 2-mercaptoehtanol; 5, R116C/T270C with 2-mercaptoehtanol; 6, R116C/T270C/G818C with 2-mercaptoehtanol. The arrow points to the monomer form (118 KDa) and upper forms represent diverse high-order forms. The quantity of the monomer form diminishes gradually in 1, 2 and 3, whereas in 4, 5 and 6 it remains constant.

An explanation for the success of this variant requires several factors to be considered simultaneously. In addition to the optimal bond distance

between Asp116 and Thr270 in the different conformations analyzed (1.80-2.25 Å), the relatively high average B-factors of these residues (34.70 and 40.98 compared with the B-factor molecule average of 19.82) make them very flexible and suitable for stabilization purposes. Therefore, the reduction in the thermal motion of these two flexible amino acids by the introduction of a new covalent bond in our design has made the area more rigid and the tetramer more stable. On the other hand, threonine is chemically analogous to cysteine; thus substitution in the position 270 does not affect other molecular contacts (Bjørk et al., 2003; Zhou et al., 2013), and hence the effects of unfavourable protein unfolding have been avoided in this way.

After obtaining the R116C/T270C mutant, which improved the thermoresistance of KlβGal by stabilizing the contacts between dimers, our rational strategy was used to reinforce the other subunit interfaces (monomer-monomer). We mutated R116C/T270C by introducing a cysteine in the 818 position. The single mutant, G818C, was the only mutation in the monomer-monomer interface that did not lower thermotolerance and catalytic activity (Table 1), which was the reason testing its possible synergistic effect of the accumulated changes in the triple mutant.

Table 1 shows that R116C/T270C/G818C retains more enzymatic activity (25% more than the native enzyme) after heat treatment than R116C/T270C (only 10% higher). This suggests an accumulative effect of the 2 mutations. SDS-PAGE analysis of purified R116C/T270C/G818C under oxidizing and reducing conditions also indicates differences from R116C/T270C. The bands corresponding to tetramer forms increase under oxidizing conditions, probably due to the formation of additional disulfide

bonds on R116C/T270C/G818C (Figure 2). The new bonds in the mutant probably total 4 in the tetramer because of the oligomerization features of KlβGal (Figure 1).

3.3. Verification of disulfide bond formation

To confirm the disulfide bonds formation, two new approaches were done: a mass spectrometry and a colorimetric assay.

By MS/MS analysis it was detected mass modifications in cysteines of the mutant variants involved in theoretical inter-chain disulfide bonds. These mass modifications are compatible with the previous existence of a disulfide bridge that was asymmetrically broken by fragmentation, resulting on the presence of dehydroalanine (dhA) in one of the two sites involved in the contact (Kim et al., 2015; Mormann et al., 2008).

In this way, both in R116C/T270C and in R116C/T270C/G818C mutants, it was detected dhA modifications in residue Cys116. Moreover, analyses showed dhA modifications in residue Cys270 of R116C/T270C/G818C variant. Thus, these data support the formation of disulfide bonds between the two cysteines introduced in the dimer-dimer interface of the enzyme.

On the other hand, in mutant R116C/T270C/G818C was detected dhA on Cys3 site, which makes a disulfide contact with Cys818 according our theoretical model. Unfortunately, peptide fragment that contains Cys818 is highly hydrophilic, which makes difficult its detection (Table 2).

Table 2: Peptide fragments with dhA modifications in cysteine sites detected by mass spectrometry analyses. Cysteines involved in disulfide bonds formation are marked in red colour.

Mutant variant	Peptide sequence	Aminoacid range	Confidence (%)
R116C/T270C	TFEL**C**SKSIESFEHR	112-126	99
R116C/T270C/G818C	DYKDDDDKS**C**LIPENLRNPK	2-13	99
R116C/T270C/G818C	TFEL**C**SKSIESFEHR	112-126	99
R116C/T270C/G818C	VYDASSLLNEENGNT**C**FSTK	255-274	97.2

Additionally, the formation of disulfide contacts in KlβGal and its mutants was also examined using DTNB [5,5´-dithiobis(2-nitrobenzoic acid)], which reacts with free sulfhydryl groups of the protein structure. The differences between the results in non-reduced and reduced conditions were significant in both mutants, R116C/T270C and R116C/T270C/G818C, while there were no significant changes between conditions in native KlβGal. Moreover, differences were more pronounced in variant R116C/T270C/G818C. These data show that R116C/T270C/G818C have a larger proportion of cysteines joined as disulfide bonds than R116C/T270C variant, as it was predicted (Table 3).

All of these results together and the improvement observed in residual activity support the presence of new disulfide contacts in the mutant enzymes obtained. The number of new disulfide bonds is higher in R116C/T270C/G818C mutant because of the additionally effect of mutation G818C, which confers new contacts in monomer-monomer interface, and in turn amplifies the residual activity improvement.

Table 3: Free sulfhydryl concentration of KlβGal and its mutants in non-reduced and reduced conditions determined by the DTNB method. The ± sign refers to standard error. *Significance p-value with non-reduce condition <0.01.

	Free sulfhydryl groups concentration (µmol/g protein)	
	Non-reduced conditions	Reduced conditions
KlβGal	0.0588±0.0199	0.0593±0.0038
R116C/T270C	0.0897±0,0046	0.1087±0.0014*
R116C/T270C/G818C	0.0862±0.0099	0.1250±0.0054*

3.4. Stability analysis of the two selected mutants

Analyses carried with highly purified protein samples, native KlβGal and the 2 selected mutants (R116C/T270C and R116C/T270C/G818C), confirmed the improved stability of the variants found during screening. Stability was tested by incubating samples at 40 and 45ºC. After incubating R116C/T270C for an hour at 40ºC, the mutant enzyme retained 45% activity, whereas the native enzyme only retained 20% of its activity (Figure 3A). After 1h at 45ºC, this mutant retained 25% of the β-galactosidase activity, but the native enzyme was almost completely (96-97%) inactivated within 20 min (Figure 3B). After 1h at 40ºC or 45^0C in R116C/T270C/G818C, the enzyme retained 79% and 49% of its initial catalytic activity, respectively (Figure 3 A and B).

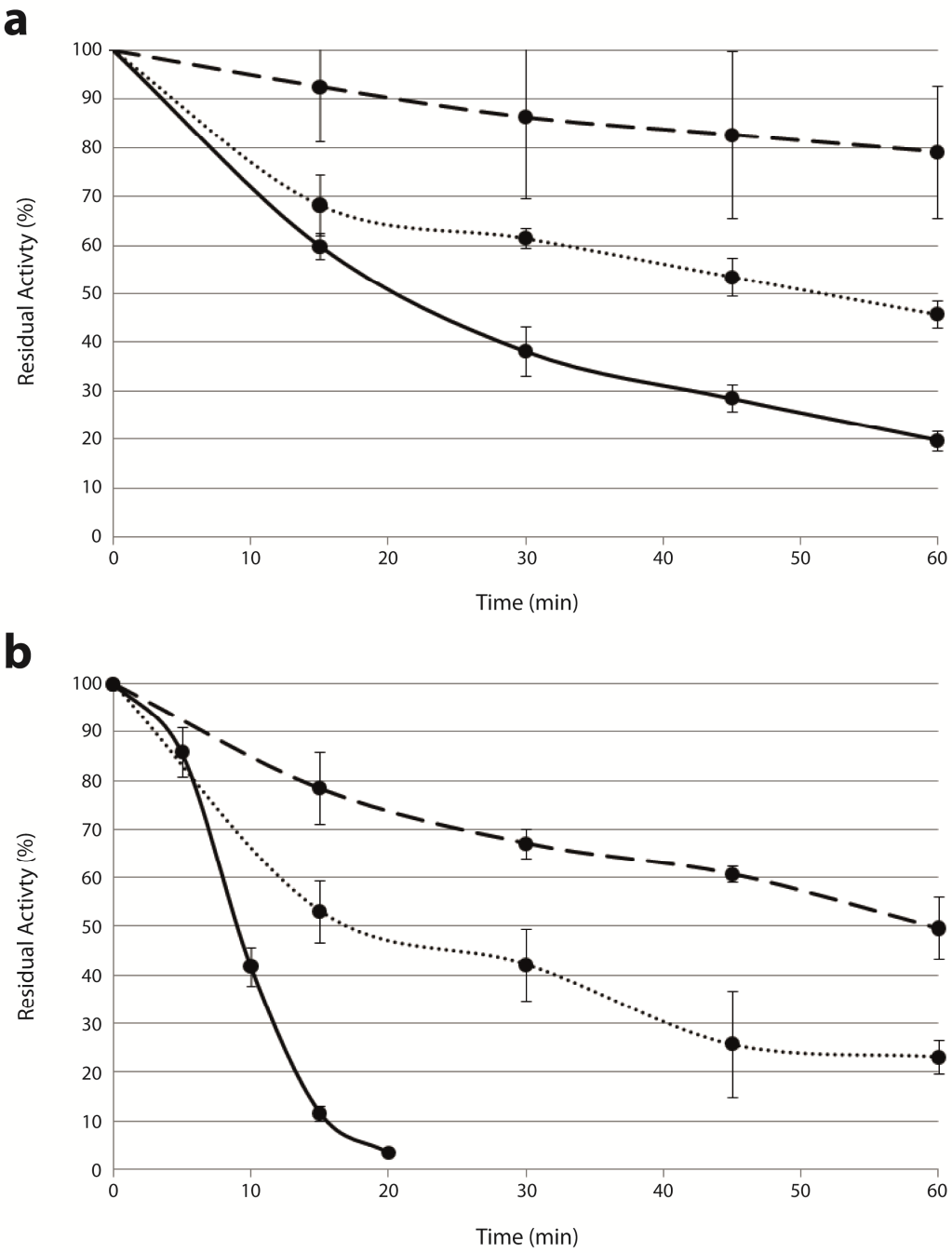

Figure 3: Effect of temperature on residual activity of each KlβGal variant. a, 40ºC treatment; b, 45ºC treatment. Solid lines represent native KlβGal, dotted lines represent R116C/T270C, and dashed lines represent R116C/T270C/G818C**.** Error bars are standard deviations of 3 measurements.

In terms of half-life at 45 ºC, KlβGal's was 26.11 min, whereas R116C/T270C and R116C/T270C/G818C half-lives were 57.64 and 178.51 min, respectively. This shows an important increase in the mutant, 2.2 fold for R116C/T270C and 6.84 fold for R116C/T270C/G818C, with reference to the native enzyme's half-life.

DSF assay of the 3 enzyme variants was used to measure and compare their Tm values. R116C/T270C (Tm=41.7 ºC) and R116C/T270C/G818C (Tm=47.8 ºC) have 2.4ºC and 8.5ºC higher values than the Tm of the native enzyme (39.3ºC) (Figure 4).

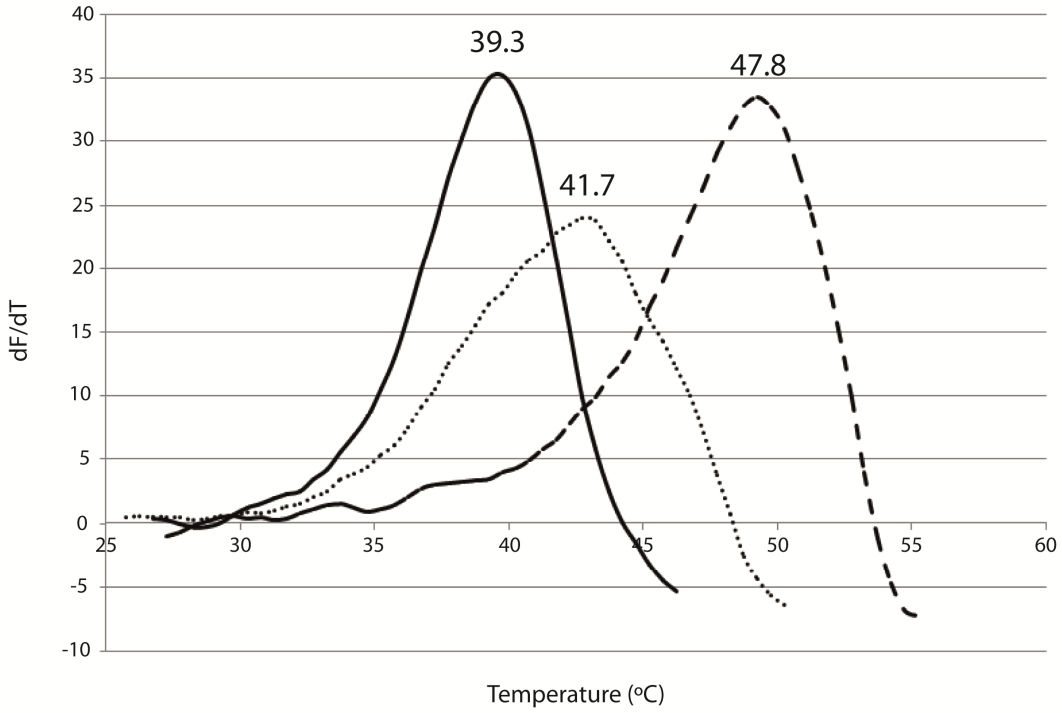

Figure 4: DSF analysis. dF/dT is plotted against temperature. The maximum of the fitted curve is the melting point (Tm) of the protein. Solid lines represent native KlβGal, dotted lines represent R116C/T270C, and dashed lines represent R116C/T270C/G818C.

These data show a significant improvement in thermostability achieved in R116C/T270C/G818C in reference to R116C/T270C, which in turn had higher thermal resistance than native KlβGal.

3.5. Kinetic analysis of hydrolytic activity in the two selected mutants

Kinetic characterization of the 2 selected variants with the artificial substrate *p*-nitrophenyl-β-D-galactopyranoside (β-PNPG) in comparison with the native form showed kinetic differences (Table 4). The 3 forms had similar affinities for this substrate, as deduced from their Km values with values between 2.15 and 2.47. However, the mutant enzymes both had a higher Vmax (99.42 U/mg for R116C/T270C and 146.3 U/mg for R116C/T270C/ G818C) than native KlβGal (71.9 U/mg). Considering that the same protein concentration had been used in all cases, the results clearly show that R116C/T270C and R116C/T270C/G818C have higher catalytic efficiency than the native protein. The differences are more pronounced in R116C/T270C/G818C, doubling values (146.3 U/mg *versus* 71.9 U/mg) found with the native enzyme (Table 4).

Table 4: Kinetic analysis of KlβGal and its mutants. The ± sign refers to standard error curve fit using the kinetic module of Prism 6.

	β-PNPG			Lactose		
	Km (mM)	Vmax (U/mg)	Vmax/Km	Km (mM)	Vmax (U/mg)	Vmax/Km
KlβGal	2.15±0.52	71.90±4.67	33.44	34.31±8.87	15.99±1.48	0.47
R116C/T270C	2.22±0.25	99.42±3.02	44.78	38.20±10.25	15.24±1.52	0.40
R116C/T270C/G818C	2.47±0.36	146.30±6.11	59.23	34.82±9.49	28.48±2.80	0.82

By kinetic analysis with lactose, a natural substrate, the affinities for the three enzymes were also similar (Km values between 34.31 and 38.20). Unexpectedly, values of Vmax of KlβGal and R116C/T270C were similar (15.99 U/mg and 15.22 U/mg), in contrast with values found with the β-PNPG substrate. However, the triple mutant, R116C/T270C/G818C, is significantly different from the other 2 enzyme forms in almost doubling their Vmax (28.48 U/mg) (Table 4).

Improvement in the Vmax of the catalytic hydrolysis by protein engineering strategy, especially remarkable in the case of R116C/T270C/G818C, has industrial applications. Processes, such as milk lactose hydrolysis or the development of whey syrups, used in human alimentation and pharmaceutical intermediates (Husain, 2010; Panesar et al., 2006; Rubio-Texeira, 2006), could benefit greatly by using this newly modified enzyme. A higher efficiency in the hydrolysis of substrates would yield the same amount of products with smaller amounts of protein, thereby reducing production costs.

3.6. Oligomerization pattern analysis

Analytical ultracentrifugation of variants under native conditions and at the highest enzyme concentration tested (0.2 mg/mL) showed differences in the oligomerization pattern (Figure 5). As before (Pereira-Rodríguez et al., 2012), the assay could only detect one of the active forms for the native enzyme, - the dimeric one - which represents 33.1% of the total. However, most of the protein was monomeric (66.9%).

R116C/T270C under the same conditions, however, had a more diverse oligomerization profile of molecular forms: monomer (21.9%), dimer (20.9%), trimer (7.9%) and tetramer (49.3%).

Finally, R116C/T270C/G818C had an oligomerization profile made up mainly of complex molecular forms, such as dimer (57.2%), trimer (12.6%) and tetramer (23%), with the monomer only representing 7.3% of the total.

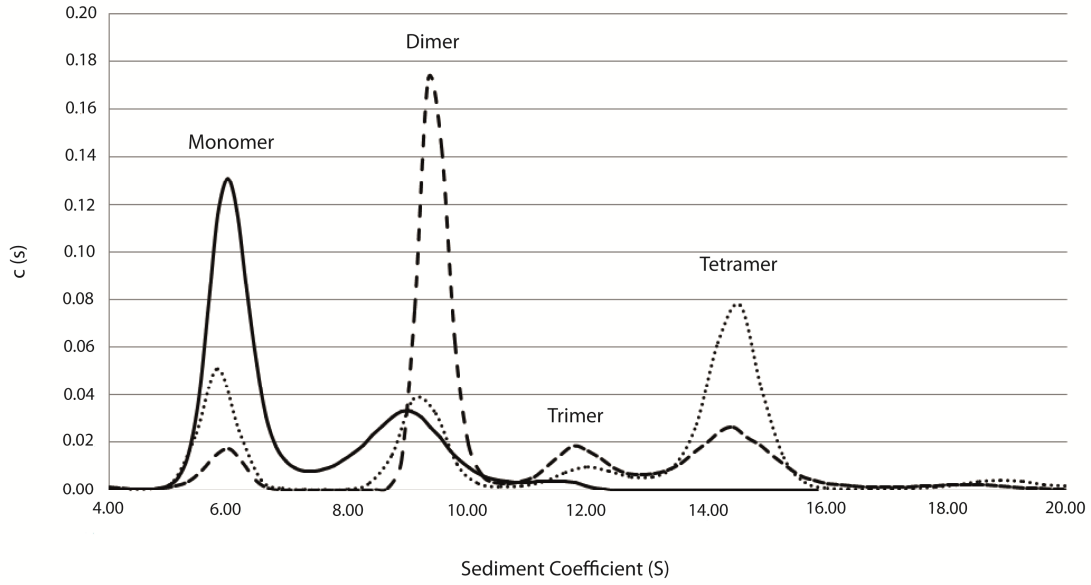

Figure 5: Analytical ultracentrifugation sedimentation velocity profile of the 3 enzyme variants (0.2 mg/mL). KlβGal (solid line), R116C/T270C (dotted line), and R116C/T270C/G818C (dashed line). Each peak is compatible with the theoretical sizes of different structural organizations: monomer, dimer, trimer and tetramer.

Using a low protein concentration (0.1 mg/mL), the results with the mutants were comparable to those obtained at a higher protein concentration, but with a differential increase in monomeric forms of 5.5 (KlβGal), 0.8 (R116C/T270C) and 2.1% (R116C/T270C/G818C), respectively, and, not unexpectedly, a concomitant lowering of enzymatic activity occurred. This suggests protein concentration and high order oligomeric species are positively correlated in the native KlβGal and its mutants. The biggest increase corresponds to the native enzyme, probably because the

initial equilibrium between oligomeric forms is displaced more towards monomers, thereby making it easier to observe this effect. Similar changes in oligomeric distribution at different protein concentrations have been observed for other proteins (Bhattacharya et al., 2014; Kutter et al., 2007; Merten et al., 2012).

Polyacrylamide Gel Electrophoresis (PAGE) in native conditions was also used to confirm these variations in the oligomer distribution (Figure 6), similar patterns being obtained as those in analytical ultracentrifugation experiments. While native enzyme is organized primarily in monomers, but showing also some oligomeric forms, both variants had considerably more oligomers. This is particularly clear in the analysis of R116C/T270C/ G818C in showing a very low proportion of monomers.

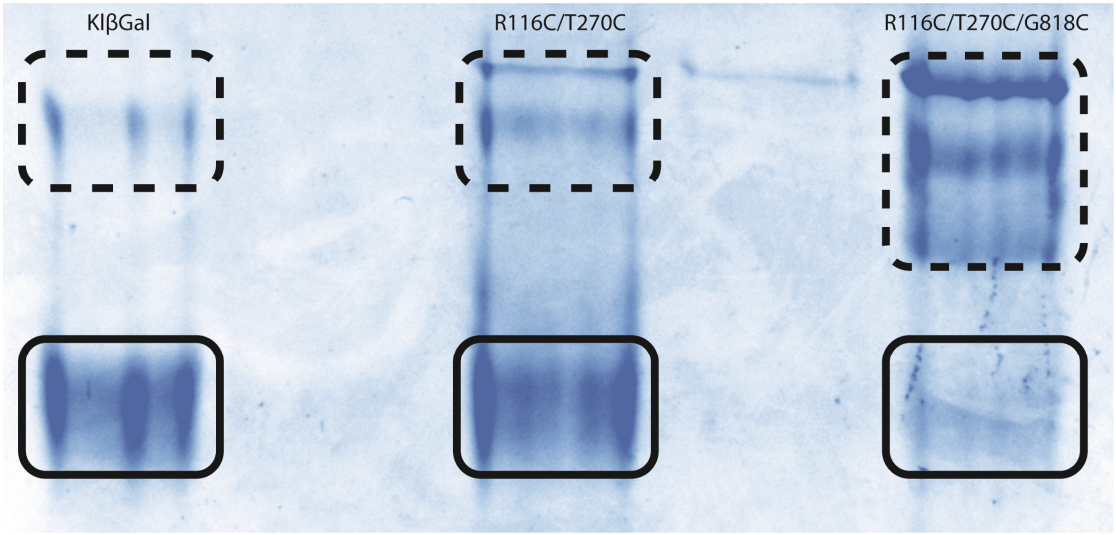

Figure 6: Native PAGE of KlβGal and its mutants. Solid line squares surround monomers and dashed line squares show oligomeric forms of each variant.

These results support the higher propensity of the variants to form stable oligomeric species than the native enzyme, thus explaining their increased enzymatic activity and stability. The increase in the proportion of active forms in R116C/T270C and R116C/T270C/G818C could explain the improved efficiency of hydrolysis by the engineered enzymes, as seen in the kinetic analysis with β-PNPG as the substrate. Likewise, the higher proportion of oligomeric forms in R116C/T270C/G818C compared with R116C/T270C explains the increase of Vmax of the triple mutant with both substrates.

The slight differences in the improvement of Vmax between KlβGal and R116C/T270C in kinetic analyses with lactose or β-PNPG as substrates (Table 4) may be due to the relatively high protein concentration required for this method. As explained above, high protein concentration is favours displacement of the equilibrium between the oligomeric forms towards higher order forms.

In conclusion, both mutants, but principally R116C/T270C/G818C, showed under all conditions tested an important improvement in activity compared with the native enzyme.

3.7. Advantages of enzyme variants for galactooligosaccharide production

Measurements of transglycosylation activity along with increased thermotolerance described above show that maximum GOS yields are higher with R116C/T270C and R116C/T270C/G818C than the native enzyme (Figure 7). Although both variants yield similar GOS production, it is noteworthy that there are the same number of enzymatic units in the reaction mixture of each of them. Therefore, taking into account the best

kinetic efficiency of R116C/T270C/G818C, this triple mutant is more suitable for this application since same GOS production is obtained with less enzyme. In fact, R116C/T270C/G818C shows more specific productivity (g GOS·mg^{-1}·h^{-1}) at 2h than R116C/T270C, 0.31 and 0.22 respectively.

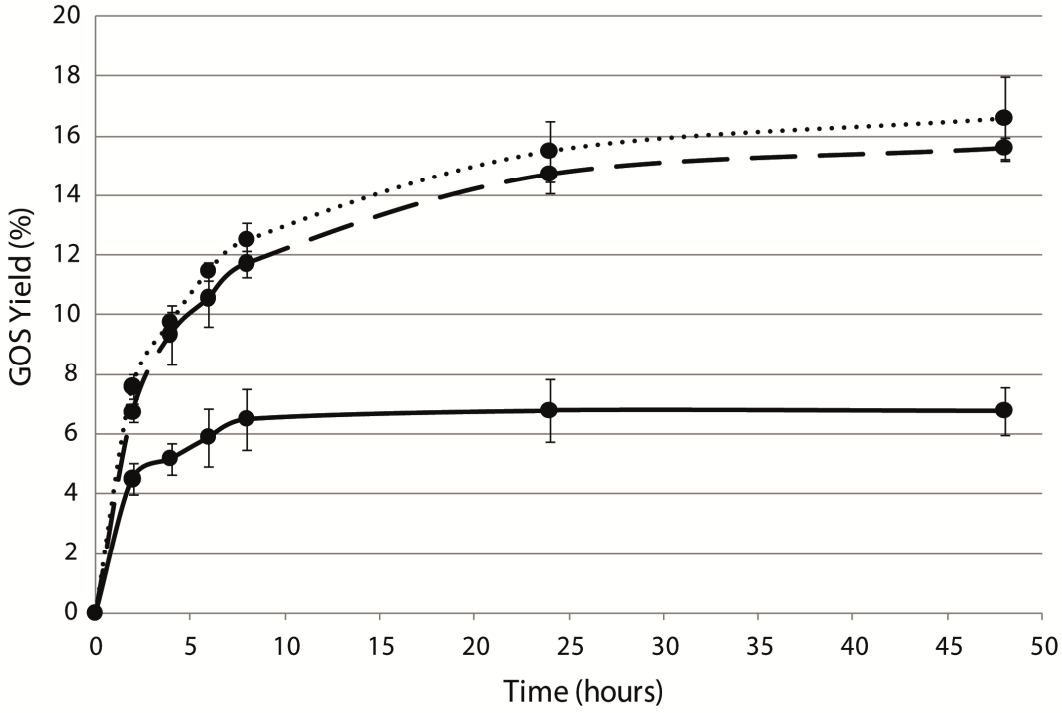

Figure 7: GOS (trisaccharides + tetrasaccharides) yield of different KlβGal variants in the synthesis assay with time. Solid lines represent native KlβGal, dotted lines represent R116C/T270C, and dashed lines represent R116C/T270C/G818C. Error bars are standards deviation of 3 measurements.

These results support the suitability of these variants for applications that involve longer incubation times of the enzyme at relatively high temperatures. For example, new strategies could be designed in the production of GOS, by using less enzyme, which represents an important saving in the processing costs. R116C/T270C/G818C would also allow the use of higher temperatures for the assay; since enzyme denaturation is

reduced, higher concentrations of lactose can be used, and consequently higher maximum yields of GOS can be achieved. The scaling-up of production and its optimization using these engineered enzymes can have significant industrial benefits.

4. CONCLUSIONS

We have engineered 2 KlβGal variants by rational mutagenesis based on the structure of the enzyme, introducing disulfide bonds in monomer-monomer and dimer-dimer interfaces. The 2 mutants, R116C/T270C and R116C/T270C/G818C, had improved thermostability as measured by residual activity after incubation at 45ºC, and also increased half-lives and Tm values compared to native enzyme under the same conditions. Kinetic parameters corresponding to the hydrolytic reaction confirmed the improvement of the catalytic activity of the 2 mutant enzymes with the artificial substrate (β-PNPG), and in the case of R116C/T270C/G818C with the natural substrate, lactose. These improvements do not affect the affinity for the substrates, but rather is a consequence of an increase in Vmax. It has been experimentally confirmed that in both mutants, but especially R116C/T270C/G818C, both these improvements correlate positively with an increase in the proportion of dimeric and tetrameric species, i.e. the active forms of KlβGal (Becerra et al., 1998).

The applicability of both mutant variants in high temperature industrial applications and interest of their use have been validated by GOS synthesis assays.

5. ACKNOWLEDGEMENTS

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Chapter 4

Preliminary immobilization studies of *Kluyveromyces lactis* β-galactosidase by using magnetic particles

SUMMARY

Immobilization methods are interesting for improving the applicability of enzymes in industry. Two kinds of magnetic particles, mPANI and mPOS-PVA, were synthetized and used to immobilize the β-galactosidase from *Kluyveromyces lactis*. The native and a thermostable mutant variant were immobilized and analyzed independently in order to compare their properties after the process. Mutant enzyme shows in both supports a reduced loss of catalytic activity after immobilization and after several reutilization cycles in comparison with the native enzyme, which suggests a better aptitude of the mutant enzyme to be immobilized. Moreover, mPOS-PVA immobilization results in all cases much more efficient in preserving enzyme activity than the mPANI method. Morphological studies show a big particle-size difference between supports, which affects the specific surface area of the particles and could explain the variation in immobilization efficiencies.

1. INTRODUCTION

Immobilization of enzymes is one of the most utilized methods to improve catalyst´s features and to reduce the costs of industrial processes. The use of diverse materials as enzyme supports has shown several advantages over soluble enzymes, such as a higher stability to changes in temperature or pH and a more easy removal of the enzyme from the reaction mixture, which allows to obtain high purified products (Homaei et al., 2013). Moreover, immobilization makes possible the reusing of the catalysts, thus producing important savings in high-scale processes.

The food industry has extensively used immobilization techniques in order to optimize catalytic reactions. Among the immobilized enzymes, β-galactosidases or lactases (3.2.1.23) stand out because of their interest in numerous applications like production of lactose-free products or synthesis of galactooligosaccharides (GOS), a group of prebiotics with tested healthy effects.

β-galactosidase from the yeast *Kluyveromyces lactis* (KlβGal) is one of the most used lactases in food industry, mainly because of its high hydrolytic activity and the safety of the producer organism, classified by FDA as GRAS (Generally Recognized as Safe). KlβGal has been immobilized by performing different strategies that include the use of supports like cotton fabric (Li et al., 2007), polymeric ultrafiltration membrane (Güleç, 2013), thiosulfinate/thiosulfonate (Ovsejevi et al., 1998) or chitosan microparticles (Klein et al., 2013).

KlβGal has also been immobilized in magnetite particles covered by the polymer polysiloxane-polyvinylalcohol (POS-PVA) (Neri et al., 2008). A similar approach with magnetic particles has been also performed to immobilize β-galactosidase from *Aspergillus oryzae*, but with magnetite polyaniline (PANI) as coverage polymer (Neri et al., 2011). An interesting property of these kind of magnetite-covered particles is their simply separation with magnetic fields, which facilitates their reutilization (Cao et al., 2012).

In the present work KlβGal was immobilized in two magnetic supports, magnetite covered by POS-PVA (mPOS-PVA) and magnetite covered by PANI (mPANI). Study of structural features of both supports and catalytic properties of the immobilized enzymes were performed in order to compare the two methods. Moreover, in parallel it was also immobilized a

KlβGal mutant (116C/270C/818C) with high thermostability and different oligomer equilibrium, to elucidate how these improved properties can affect the immobilization process of the enzyme.

2. MATERIAL AND METHODS

2.1. Supports synthesis

To obtain POS-PVA beads, the method described by Barros et al. (Lima Barros et al., 2002) was employed. 5 mL of ethanol, 5 mL of tetraethylorthosilicte (TEOS) and 6 mL of 2% (w/v) of polyvinyl alcohol were mixed. The mixture was heated to 60 ºC and after the addition of concentrated HCl, it was incubated for 5 min at this temperature. Later, solution was allocated into ELISA microwell plates. After 72 h at room temperature, the resulting solidified beads were smashed to powder and resuspended in 100 mL of deionized water.

In order to magnetize the support, 10 mL of a solution 1:1 of 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ was added slowly to the powder solution, heating up the mixture on a magnetic stirrer. After the pH was adjusted to 11 with NH_4OH, the beaker contents were incubated at 100 ºC for 30 min. Particles were washed with deionized water until the pH dropped to 7. Finally the particles were dried overnight at 60ºC and smashed to powder with mortar and pestle.

mPANI particles were obtained using the coprecipitation method. Magnetite was obtained by the same procedure described in POS-PVA synthesis. After overnight driying of the magnetite particles, they were mixed with 4 mL of $KMnO_4$ 0.1 M and incubated for 1 hour at room temperature**.** Subsequently, particles were washed with deionized and dissolved in a 0.5 M solution of aniline prepared in 1 M HCl. After 30 min

incubation, particles were washed consecutively with deionized water (5x), HCl 2M (5x) and deionized water (5x). Finally, particles were dried overnight at 60 ºC.

2.2. β-galactosidase immobilization

To activate the supports, 10 mg of POS-PVA particles and mPANI particles were incubated in 10 mL of a 0.25 % w/v gluteraldehyde solution, prepared in 0.1 M H_2SO_4 (POS-PVA particles)or 20 mM citrate-phosphate buffer, pH 4.5 (mPANI particles). The mixture was incubated for 2 h at room temperature in a rotator stirrer. The particles were washed (10 x) with deionized water. 0.2 mg of purified protein (native KlβGal and thermostabilized mutant) diluted in 1 mL of TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.4) were added and immobilized for 18 hours at 4ºC in a rotator stirrer. After incubation, particles were washed 5 times with Z buffer (100 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.6 mM $MgSO_4$) before activity and immobilization efficiency tests.

2.3. β-galactosidase activity

Lactose hydrolysis in different samples was tested by measuring glucose production. Glucose concentration was measured using the commercial kit D-Glucose GOD-POD (Nzytech), following the manufacturers' recommendations.

The hydrolytic activity of immobilized and free enzyme was compared in order to test the immobilization efficiency. All the samples were diluted in 2 mL of Z buffer. The reaction times were 5-20 minutes and the reaction temperature was 30 ºC. The reaction was stopped by heating samples at 96 ºC for 5 min.

2.4. Reuse of immobilized enzyme

Retention of hydrolytic activity of immobilized enzymes was tested by measuring the catalytic ability of the same particles in 10 consecutive cycles. Particles were washed three times with Z buffer between cycles and activity measures were performed following the method described above.

2.5. Support characterization

SEM analyses were made by examination of samples with ultrahigh resolution microscope Nova Nano SEM 200. Elemental analyses of samples were performed with an integrating system EDS/EBSD EDAX-Pegasus X4M.

3. RESULTS AND DISCUSSION

3.1. Immobilization efficiency and reuse of immobilized enzyme

Lactose hydrolysis measurements exhibit significant changes in immobilization efficiencies between enzyme variants in both supports. Moreover, there are important differences in immobilization efficiencies depending on the magnetic particle utilized as support.

KlβGal immobilized in mPOS-PVA maintains 15.85% of the hydrolytic activity of free enzyme, while mutant 116C/270C/818C preserves 28.08 %. In mPANI support, immobilization efficiency with KlβGal results only 2.10 %. Immobilization efficiency of mutant 116C/270C/818C in this support doubles native enzyme result by achieving 4.33 % (Table 1).

Table 1: Immobilization efficiency of different methods performed.

Enzyme variant	Immobilization support	% Specific activity immobilized vs free enzyme
KlβGal	mPOS-PVA	15.85±0.32
116C/270C/818C	mPOS-PVA	28.08±1.61
KlβGal	mPANI	2.10±1.23
116C/270C/818C	mPANI	4.33±0.66

Results show that 116C/270C/818C mutant preserves a bigger proportion of active enzyme after immobilization than KlβGal in the two methods tested. Moreover, mPOS-PVA particles are much more efficient than mPANI particles in maintaining the catalytic activity of the enzymes.

Reutilization experiments show differences between enzyme variants and between immobilization supports.

Regarding immobilization methods, mPANI maintains a bigger proportion of enzyme activity than mPOS-PVA along consecutive cycles, preserving more enzymatic activity after 10 cycles, by using both of the protein variants (Figure 1). However, differences are too small to counteract the big gap between the immobilization efficiency of the two supports.

Concerning the two protein variants, mutant enzyme preserves a bigger proportion of enzyme activity along reutilization cycles than native enzyme. Moreover, results show that there are differences between enzyme variants regardless of the utilized support. Thus, while native enzyme maintains 26.64 % of initial hydrolytic activity after cycle 10 on mPANI support and 16.73 % on mPOS-PVA support, mutant variant

preserved 39.91 % of initial activity on mPANI support and 33.45 % on mPVA-POS particles (Figure 1).

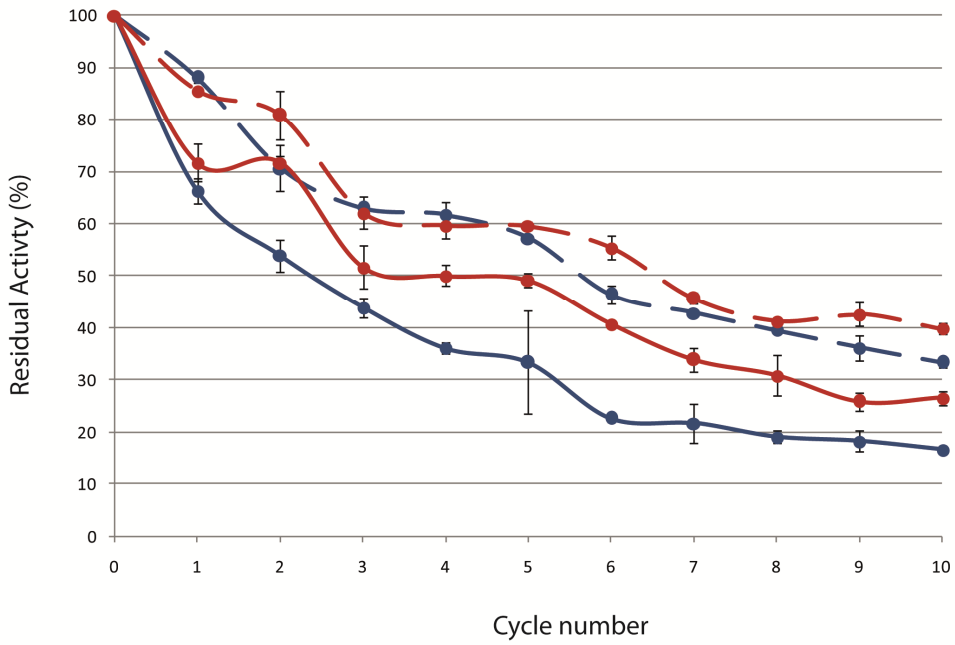

Figure 1: Effect of the number of reutilization cycles on the activity of the immobilized enzyme. Red solid line represents native Kl-β-Gal immobilized with mPANI particles, red dashed line represents R116C/T270C/G818C mutant immobilized with mPANI particles, blue solid line represents native Kl-*β*-Gal immobilized with mPOS-PVA particles and blue dashed line represents R116C/T270C/G818C mutant immobilized with mPVA-POS particles. Error bars are standards deviation of 3 measurements.

The results of both immobilization efficiency and reutilization experiments denote the better performance of 116C/270C/818C variant for immobilization in the two selected supports. The superiority of the mutant enzyme over native enzyme is probably caused by the intrinsic structural features of this genetically modified variant. As previously reported (Chapter 3 of this work), the presence in this variant of disulphide contacts between subunits produces an increase in the proportion of active oligomeric forms of the enzyme (dimers and tetramers). This could

promote in 116C/270C/818C the preferent immobilization of more active forms. Moreover, the covalent nature of disulphide bonds makes these catalytic active oligomers of the 116C/270C/818C variant more resistant to the spontaneous cleavage into monomers (inactive forms), which explains the better immobilization efficiency and reutilization of the mutant enzyme in both immobilization supports.

3.2. Support characterization

The two particle types were analyzed by SEM (*Scanning Electron Microscopy*). The results of microscopic characterization show that mPANI particles have a variable size between 20 and 200 µm (Figure 2A). Furthermore, the particles have a uniform and smooth surface (Figure 2B). Although the mPOS-PVA particles apparently had similar size to mPANI particles (Figure 2C), a detailed analysis of them showed a nanoparticle coating on the magnetite, which confers them a more irregular surface (Figure 2D).

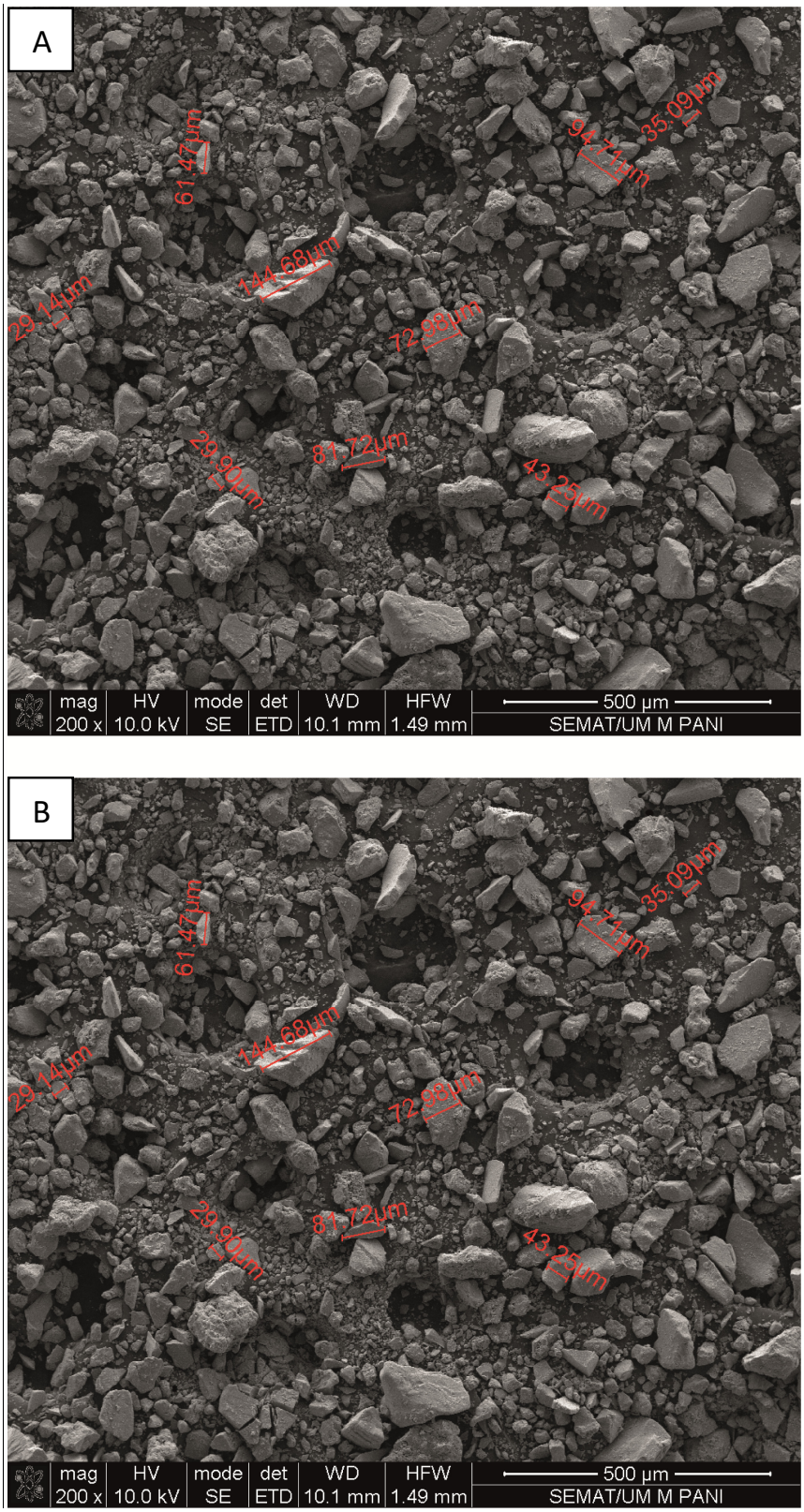

Figure 2: SEM images of mPANI and mPOS-PVA particles. A) General view of mPANI particles sample; B) Detailed view of mPANI particles sample.

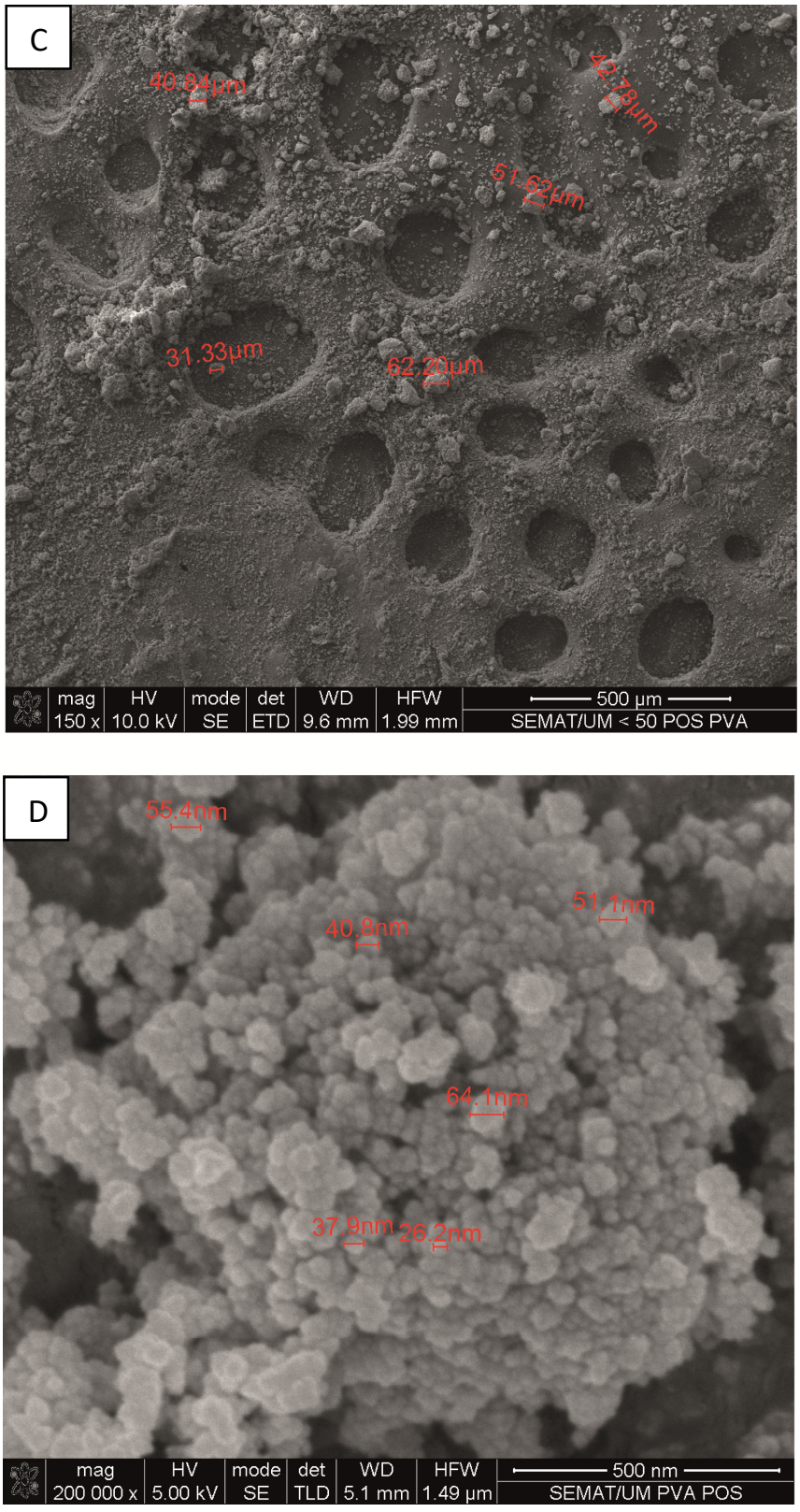

Figure 2: SEM images of mPANI and mPOS-PVA particles (continuation). C) General view of mPOS-PVA particles sample; D) Detailed view of mPOS-PVA particles sample.

Elemental analysis of the mPOS-PVA particles in the support with higher immobilization efficiency confirmed de presence of magnetite and polymer elements, which supports the correct synthesis of them (Figure 3).

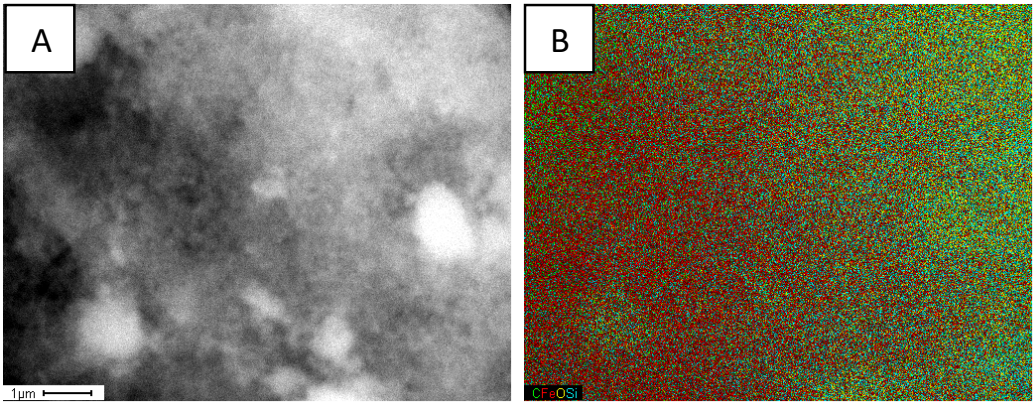

Figure 3: Elemental analysis of mPOS-PVA particle surface; A) SEM-Atomic number contrast image; B) SEM-Elemental colour mapping image.

The characterization of the supports reveals important differences in their morphological properties. The nanometric size of mPOS-PVA particles could explain that they are much more adequate for the immobilization of KlβGal and 116C/270C/818C than mPANI particles. It was reported that nanoparticles have high enzyme loading capability due their large specific surface areas in comparison with microparticles (Cao et al., 2012).

However, although much less accentuated with mPOS-PVA support, there is an important loss of hydrolytic activity after carrying out the two immobilization techniques. This might be caused by diffusion limitations promoted by immobilization methods that could difficult the availability of the substrate in the support and therefore affect the catalytic performance of the enzyme (Garcia-Galan et al., 2011; Santos et al., 2015).

Results of this work support the better aptitude of 116C/270C/818C mutant to be immobilized in two magnetic supports in comparison with

KlβGal. It would be interesting to carry out studies with other supports in order to confirm that this advantage of the mutant variant is consistent. It would imply that variant 116C/270C/818C had another interesting feature for its use in industrial applications, to add to previously improved properties already reported in chapter 3.

Concluding Remarks

The main conclusions from the studies reported in the present thesis can be summarized as follows:

1. We have purified and crystallized the β-galactosidase from *Aspergillus niger* rod-shaped crystals grew with PEG 3350 as main precipitant agent. A diffraction data set was collected to 1.8 Å resolution.
2. We have solved the structure of β-galactosidase from *Aspergillus niger* and its complex with the oligosaccharides allolactose, 3-galactosyl-glucose, 6-galactosyl-galactose, 4-galactosyl-lactose and 6-galactosyl-lactose.
    2.1. As other fungal GH35 β-galactosidases, AnβGal is monomeric and folds into six domains with a horseshoe disposition.
    2.2. Residues Phe264, Tyr304 and Trp806 make a dynamic hydrophobic platform that accommodates the sugar at subsite +1, suggesting an important role in the recognition of structurally different substrates. Mutagenesis analyses of residues Tyr304 and Trp806 support the importance of the hydrophobic contacts made by these amino acids with the substrate for the hydrolytic function of the enzyme.
    2.3. Complexes with the trisaccharides show two potential subsites at +2 position, depending on the substrate type. This feature and the wide cavity housing the active site of the enzyme suggest that it might also accommodate branched substrates.
3. We have generated mutants of β-galactosidase from *Aspergillus niger* with improved transgalactosylation activity by selecting mutagenesis targets by the structural comparison of the enzyme with other GH35 β-galactosidases.

3.1. The introduction of amino acids that promote a more hydrophobic environment results in a higher tendency of sugars *vs.* water to be acceptors and, consequently, in an increased transgalactosylation ability.

4. Using rational mutagenesis we have obtained two variants of β-galactosidase from *Kluyveromyces lactis* by introducing disulfide bonds in monomer-monomer and in dimer-dimer interfaces.

4.1. The two mutant enzymes showed higher thermostability and increased their half-life at 45 ºC 2.2 and 6.8 times respectively, compared to the obtained for the wild type enzyme in the same conditions. Moreover, melting temperature values were 2.4 and 8.5 ºC respectively higher than the value obtained with the wild time enzyme.

4.2. The enrichment in enzymatically active oligomeric forms in these mutants increases their catalytic efficiency, due to the reinforcement of the interface contacts achieved by the introduction of disulfide bonds.

4.3. GOS synthesis assays support the applicability of both mutants for industrial applications requiring high temperature.

5. We have also performed preliminary immobilization tests with *Kluyveromyces lactis* β-galactosidase and the most thermostable mutant obtained in two different magnetic supports. These studies show:

5.1. mPVA-POS support resulted more adequate than mPANI support to immobilize both enzyme variants. This could be explained by the smaller size of mPVA-POS particles, which implies an increase in the specific surface area for immobilization.

5.2. Results also reveal the increased ability of the thermostable *Kluyveromyces lactis* β-galactosidase obtained to be immobilized. This is supported by the significant improvement in immobilization efficiency and in reutilization capacity of the mutant variant in comparison with the native enzyme in both supports.

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Appendix I

Resumen

1. INTRODUCCIÓN

Las enzimas han sido usadas desde la antigüedad en la elaboración de muchos alimentos (Vasic-Racki, 2006), siendo en la actualidad muy utilizadas en procesos industriales (Jemli et al., 2016). En el sector lácteo, unas de las enzimas más usadas son las β-galactosidasas, que hidrolizan o transglicosilan β-galactósidos dependiendo de las condiciones iniciales de la reacción (Husain, 2010; Oliveira et al., 2011).

Las β-galactosidasas se clasifican dentro de la clase enzimática de las glicosil-hidrolasas (GH), encontrándose representadas en varias familias dentro de esta clase (GH1, GH2, GH3, GH35, GH43, GH50 y GH59) (Cantarel et al., 2009).

La actividad catalítica de estas enzimas se lleva a cabo por un mecanismo de retención, en el que el producto final de la reacción depende de la naturaleza de la molécula que actúa como aceptora en el segundo paso de la reacción. De esta forma, si la molécula aceptora es agua se producirá una reacción hidrolítica, y si es un galactósido se producirá una reacción de transgalactosilación (Sangwan et al., 2011). Uno de los factores que más influyen en la preferencia de la enzima por uno u otro aceptor es la concentración inicial de cada una de las moléculas aceptoras (Gosling et al., 2010).

En esta tesis se trabajó con dos de las β-galactosidasas más usadas tradicionalmente en la industria alimentaria, la procedente del hongo filamentoso *Aspergillus niger*, y la procedente de la levadura *Kluyveromyces lactis*. Ambas han sido muy usadas debido a características favorables como su alta capacidad hidrolítica y su procedencia de

organismos reconocidos generalmente como seguros (GRAS) (Schuster et al., 2002; Panesar et al., 2006).

La β-galactosidasa de *A. niger* es una enzima con pH óptimo ácido, que se suele usar para la hidrólisis de la lactosa de sueros ácidos obtenidos como residuo en las industrias queseras (Panesar et al., 2006). Algunos de los atractivos de esta enzima para su uso en aplicaciones industriales son su expresión extracelular y su relativa termoestabilidad. La β-galactosidasa de *A. niger* pertenece a la familia GH35 y la resolución de la estructura tridimensional de otras enzimas similares muestra una disposición monomérica formada por seis dominios (Maksimainen et al., 2011; Maksimainen et al., 2013; Rojas et al., 2004). Sin embargo, las características catalíticas y las preferencias por distintos aceptores varían entre esta β-galactosidasa y las del resto de las enzimas similares caracterizadas estructuralmente hasta ahora (Dragosits et al., 2014). El estudio de la estructura de esta enzima contribuye por tanto a dilucidar las claves de estas diferencias.

Por su parte, la β-galactosidasa de *K. lactis*, debido a su alta actividad a pH neutro se ha usado extensamente para la hidrólisis de la lactosa de la leche (Husain, 2010; Rubio-Texeira, 2006). Sin embargo, su uso en ciertas aplicaciones, como es el caso de la síntesis de GOS, se ve limitado por su labilidad térmica. La β-galactosidasa de *K. lactis* es codificada por el gen *LAC4*, pertenece a la familia GH2 y tras cristalización se ha caracterizado su estructura tetramérica formada por dos dímeros idénticos (Pereira-Rodríguez et al., 2012). En solución seen encuentra en un equilibrio de distintas formas oligoméricas (monómero, dímero y tetrámero) siendo los dímeros y las formas oligoméricas superiores las que poseen actividad enzimática (Becerra et al., 1998).

Las β-galactosidasas se han empleaado en varios tipos de aplicaciones industriales. La más conocida es su utilización en la producción de alimentos sin lactosa (Neri et al., 2008) que permite el consumo de leche y sus derivados por una gran parte de la población mundial que no es capaz de digerir la lactosa al reducirse los niveles de expresión de la enzima lactasa después de los primeros meses de vida (Ingram et al., 2009; Itan et al., 2010). La capacidad hidrolítica de las β-galactosidasas también se ha usado para mejorar las propiedades fisicoquímicas y organolépticas de ciertos productos, al aumentar por ejemplo su solubilidad y su dulzor (Dutra Rosolen et al., 2015); evitando a su vez muchos defectos de los productos lácteos refrigerados, como la formación de depósitos, textura granulosa y cristalización (Panesar et al., 2007). Estas enzimas también han sido utilizadas para transformar el suero ácido de la leche, un residuo industrial, en un suero dulce útil para suplementar ciertos alimentos (Panesar et al., 2006).

Además de los usos derivados de la actividad hidrolítica, la actividad de transgalactosilación de las β-galactosidasas permite la obtención de galactooligosacáridos (GOS), moléculas prebióticas con interés creciente en la industria alimentaria y farmacéutica (Lamsal, 2012; Rastall, 2010; Roberfroid, 2007). Se ha verificado en varios trabajos la potencialidad de los GOS para ser usados en el enriquecimiento de leches artificiales para lactantes (Sangwan et al., 2011; Vandenplas et al., 2015) o para producir efectos beneficiosos en la modulación del sistema inmunitario o en el tratamiento de ciertos desórdenes como la alergia, el asma o el cáncer colorrectal (Bruno-Barcena and Azcarate-Peril, 2015; Lamsal, 2012; Sangwan et al., 2011).

La estructura de los GOS varía en aspectos como el tipo de enlace y el nivel de oligomerización, dependiendo de la enzima utilizada. De esta forma, por ejemplo, las β-galactosidasas del género *Aspergillus* o de *K. lactis* tienden a producir una mayor proporción de trisacáridos con enlaces de tipo β (1-6) (Rodriguez-Colinas et al., 2011; Urrutia et al., 2013), mientras que otra enzima muy utilizada en la industria alimentaria, la β-galactosidasa de *Bacillus circulans*, tiende a producir una mayor cantidad de trisacáridos, pero en este caso con enlaces de tipo β (1-4) (Rodriguez-Colinas et al., 2012).

El uso de enzimas en aplicaciones industriales suele estar limitado por sus características intrínsecas, que pueden variar con respecto a las deseadas para llevar a cabo un determinado proceso. Ante este problema se han planteado varias soluciones, entre las que se encuentra la modificación de la enzima mediante técnicas de ingeniería genética (Davids et al., 2013; Yang et al., 2014). Si el conocimiento de la estructura de la enzima estádisponible, se suelen usar métodos como la mutagénesis dirigida; este sistema tiene un carácter más racional que otros métodos como la evolución dirigida y presenta ciertas ventajas como su alta eficiencia y la reducción del número de posibles candidatos a verificar (Yang et al., 2014). Otra de las técnicas más comúnmente usada para mejorar las prestaciones de las enzimas en la industria (estabilidad, selectividad o tolerancia al sustrato entre otras) es su inmovilización en un determinado soporte. Además, este tipo de técnicas permite la reutilización de las enzimas y facilita su separación del producto de reacción, lo que implica un ahorro en los costes del proceso (Polizzi et al., 2007).

2. OBJETIVOS Y METODOLOGÍA

El principal objetivo de esta tesis es estudiar y mejorar las características de dos enzimas ampliamente usadas en la industria alimentaria, las β-galactosidasas de *A. niger* y de *K. lactis*.

Para dilucidar las claves que definen las propiedades de las enzimas, es necesario tener un conocimiento profundo de sus estructuras. Por ello, se llevó a cabo la caracterización estructural de la β-galactosidasas de *A. niger*. El primer paso para esta caracterización es la purificación, expresión y cristalización de la enzima, que se detalla en el primer capítulo. Posteriormente, en el segundo capítulo, se describe la determinación de la estructura de la proteína mediante difracción de rayos X de los cristales obtenidos, usando el método de reemplazamiento molecular. Se describe además, la resolución de las estructuras de la proteína en la forma nativa y acomplejada con diferentes azúcares (disacáridos y trisacáridos) que pueden funcionar a la vez como sustratos y productos de la enzima. El estudio de las interacciones de estos azúcares en el centro activo, así como la comparación con otras enzimas de la familia, nos permitió la obtención mediante mutagénesis dirigida de variantes enzimáticas mutantes con nuevas características interesantes para su uso en la industria.

En el tercer capítulo, se detalla la modificación mediante mutagénesis dirigida de la β-galactosidasa de *K. lactis*, con el fin de mejorar propiedades como termoestabilidad y eficiencia catalítica de la enzima. Para ello, se utilizó la información estructural disponible de la enzima para diseñar mutaciones que reforzasen mediante nuevos contactos las superficies de interacción de las subunidades de la proteína.

Finalmente, en el cuarto capítulo se describe la comparación de dos métodos de inmovilización mediante partículas magnéticas de la β-galactosidasa de *K. lactis*, comparando a su vez el comportamiento de la enzima nativa y de la mutante con las mejores características descrita en el capítulo anterior.

3. RESULTADOS

3.1. Caracterización estructural y obtención de nuevas variantes de β-galactosidasa de *A. niger*

La enzima se expresó en levaduras y se purificó mediante cromatografía de afinidad para la obtención de material apropiado para llevar a cabo los experimentos de cristalización. Además, se desglicosiló la enzima recombinante mediante endoglucosidasa H para reducir la heterogeneidad de la muestra y facilitar su cristalización, resultando la masa molecular de la proteína desglicosilada en 109 kDa. Utilizando esta enzima purificada, se obtuvieron cristales adecuados para ser difractados por rayos X.

Los datos obtenidos en la difracción de estos cristales proteicos permitieron la resolución de la estructura tridimensional de la enzima. La estructura resultó ser muy similar en sus características (disposición de dominios y oligomerización) a otras estructuras de proteínas homólogas de la familia GH35. Se trata por tanto de una enzima monomérica en la que cinco dominios β-sandwich se disponen alrededor de un dominio central catalítico de tipo barril $(\alpha/\beta)_8$ distorsionado. Así mismo, como en el caso del resto de β-galactosidasas fúngicas de la familia caracterizadas estructuralmente, se detectaron varios puntos de glicosilación, entre los que destacan los aminoácidos Asn373, Asn622 y Asn914, que mantienen

intactas largas cadenas de glucosilaciones a pesar de la digestión de la enzima con endoglucosidasa H.

Por otra parte, la resolución de la estructura tridimensional acomplejada con diferentes oligosacáridos (6-galactosil-glucosa, 6-galactosil-galactosa, 3-galactosil-glucosa, 4-galactosil-lactosa y 6-galactosil-lactosa) permite ilustrar los determinantes estructurales de la amplia especificidad de la enzima ante diferentes tipos de enlaces glucosídicos [β(1-3), β(1-4) y β(1-6)]. En relación a esto, las diferencias en las configuraciones de los residuos Phe264, Tyr304 y Trp806 en varios de los complejos evidencian la formación de una plataforma hidrofóbica dinámica que acomoda el azúcar en el subsitio +1;esto sugiriere un papel principal de estos residuos en el reconocimiento de sustratos estructuralmente diferentes. Además, los complejos con trisacáridos muestran dos subsitios +2 potenciales, dependiendo del tipo de sustrato. Esta característica y la peculiar forma de su amplia cavidad en el centro activo sugieren que la enzima podría acomodar compuestosramificados de la compleja red de polisacáridos que componen el material vegetal yque sirve como sustratos de la enzima en la naturaleza.

A partir de estos estudios estructurales se seleccionaron aminoácidos potencialmente relevantes para la actividad de la enzima y se llevaron a cabo análisis mediante mutagénesis dirigida para evaluar el papel de éstos en el funcionamiento catalítico y en el ratio actividad hidrolasa/actividad transferasa. Estos datos, así como estudios comparativos de la secuencia aminoacídica de la enzima con respecto a la secuencia de otras β-galactosidasas fúngicas de la misma familia, sirvieron como base para la obtención de mutantes con una actividad de transgalactosilación significativamente superior a la registrada en la variante nativa. En

particular, la variante Y304F/Y355H/N357G/W806F produce una mayor cantidad de GOS que la β-galactosidasa de *A. oryzae* que es la enzima más usada en la industria por su alta actividad transferasa. La explicación de esta capacidad transgalactolítica aumentada podría deberse a la presencia en los mutantes de aminoácidos con un carácter más hidrofóbico, lo que favorece la utilización de otros azúcares, en vez de moléculas de agua, como aceptores de la galactosa después de la primera fase de la reacción catalítica.

En conjunto, estos resultados proporcionan nuevos conocimientos de los determinantes que modulan la especificidad y la capacidad catalítica de las β-galactosidasas fúngicas de la familia GH35. Esto sirve como base fundamental para la obtención de nuevas herramientas para la futura mejora de estas enzimas, las cuales son una interesante diana para el diseño racional de nuevos catalizadores optimizados para su uso en la industria alimentaria.

3.2. Obtención de variantes termoestables de β-galactosidasa de *K. lactis* y aproximación a su inmovilización mediante diferentes métodos.

La β-galactosidasa de *K. lactis* ve limitado su uso en aplicaciones que requieren altas temperaturas, como es el caso de la síntesis de GOS. Para la obtención de nuevas variantes termoestables, se siguió una estrategia de carácter racional, introduciendo puentes disulfuro en las superficies de interacción entre las subunidades de la enzima, tanto en la superficie de interacción entre dos monómeros, como en la superficie de interacción de los dos dímeros. Después de generar los mutantes mediante técnicas de mutagénesis dirigida, se verificó la efectiva formación de los puentes disulfuro por métodos electroforéticos, colorimétricos y de espectrometria

de masas. Los dos mutantes obtenidos, R116C/T270C y R116C/T270C/G818C, presentan vidas medias a 45 ºC 2,2 y 6,8 veces superiores respecto a la registrada para la forma nativa. Del mismo modo, los valores de las temperaturas de fusión de R116C/T270C y R116C/T270C/G818C son 2,4 y 8,5ºC más altas que la temperatura de fusión de la enzima silvestre. Mediante técnicas de ultracentrifugación analítica y electroforéticas, se verificó el enriquecimiento en formas oligomericas activas (dímeros y formas superiores) en estas variantes mutantes debido al refuerzo de los contactos de las superficies de interacción entre las subunidades, lo que también produce un incremento de su eficiencia catalítica. De esta forma, los valores de velocidad de hidrólisis máxima del sustrato artificial *p*-nitrofenil-β-D-galactopiranosido son 1,4 (R116C/T270C) y 2 (R116C/T270C/G818C) veces más altos que los de la enzima nativa. Por su parte, usando lactosa como sustrato, la velocidad de hidrólisis máxima de R116C/T270C/G818C casi dobla la velocidad máxima de la β-galactosidasa sin mutar. Las enzimas mutantes se validaron a escala de laboratorio para su uso en aplicaciones que dependen de tiempos prolongados de incubación a altas temperaturas, mediante la monitorización de sus actividades catalíticas en la síntesis de GOS, confirmándose su potencial para su utilización en este tipo de procesos industriales.

Para inmovilizar la β-galactosidasa de *K. lactis*, se sintetizaron dos clases de partículas magnéticas, magnetita recubierta con polianilina (mPANI) y magnetita cubierta de un polímero de polisiloxano y alcohol polivinílico (mPOS-PVA). Se inmovilizaron tanto la enzima nativa como el mutante termoestable obtenido que tenía mejores propiedades

(R116C/T270C/G818C) y se analizaron independientemente para comparar sus características tras el proceso. La enzima mutante muestra en ambos soportes una menor pérdida de actividad catalítica después de la inmovilización y después de varios ciclos consecutivos de reutilización en comparación con la enzima nativa, lo que sugiere una mejor aptitud de la variante mutante para ser inmovilizada. Además, la inmovilización con mPOS-PVA resultó en todos los casos mucho más eficiente a la hora de preservar la capacidad catalítica de la enzima que el método de inmovilización con mPANI. Los estudios morfológicos mediante microscopía electrónica de barrido muestran una gran diferencia en el tamaño de partícula entre los soportes, lo que afecta al área superficial específica de las partículas y podría explicar la variación de las eficiencias de inmovilización.

4. CONCLUSIONES

Las principales conclusiones de los estudios llevados a cabo se resumen en los siguientes puntos:

1. Hemos purificado y cristalizado la β-galactosidasa de *A. niger*. Se produjo el crecimiento de cristales en forma de barra, usando PEG 3350 como principal agente precipitante. Se obtuvo un conjunto de datos de difracción a una resolución de 1.8 Å.
2. Hemos determinado la estructura de la β-galactosidasa de *A. niger* y sus complejos con los oligosacáridos alolactosa, 3-galactosil-glucosa, 6-galactosil-galactosa, 4-galactosil-lactosa y 6-galactosil-lactosa.
    2.1. Tal y como sucede con otras β-galactosidasas fúngicas de la familia GH35, la β-galactosidasa de *A. niger* es una enzima

monomérica, formada por seis dominios organizados en una disposición en forma de herradura.

2.2. Los residuos Phe264, Tyr304 y Trp806 forman una plataforma hidrofóbica que acomodan al azúcar en el subsitio +1, sugiriendo un papel importante en el reconocimiento de sustratos estructuralmente diferentes. Los análisis de mutagénesis de los residuos Tyr304 y Trp806 apoyan la importancia de los contactos hidrofóbicos hechos por estos aminoácidos con el sustrato en la correcta función hidrolítica de la enzima.

2.3. Los complejos con los trisacáridos muestran dos subsitios +2 potenciales, dependiendo del tipo de sustrato. Esta característica y la amplia cavidad que aloja al centro activo de la enzima sugieren que ésta podría acomodar sustratos ramificados.

3. Hemos obtenido mutantes de β-galactosidasa de *A. niger* con mayor actividad de transgalactosilación, seleccionando dianas de mutagénesis mediante la comparación estructural de la enzima con otras β-galactosidasas de la familia GH35.

3.1. La introducción de aminoácidos que promueven un ambiente más hidrofóbico produjo una mayor tendencia de los azúcares respecto al agua a ser aceptores y, por consiguiente, una mayor capacidad transgalactolítica.

4. Hemos obtenido mediante mutagénesis racional dos variantes de la β-galactosidasa de *K. lactis* basándonos en la estructura de la enzima, introduciendo puentes disulfuro entre las superficies de interacción entre monómeros y entre dímeros.

4.1. Las dos enzimas mutantes presentan una mayor termoestabilidad, incrementando sus vidas medias a 45 ºC 2,2 y

6,8 veces respectivamente en comparación con la obtenida para la enzima silvestre en las mismas condiciones. Además, las temperaturas de fusión superaron en 2,4 y en 8,5 ºC respectivamente el valor obtenido para la variante silvestre.

4.2. El aumento en la proporción de especies oligoméricas con actividad enzimática en estas variantes mutantes incrementa la eficiencia catalítica, debido al fortalecimiento de los contactos entre subunidades producido mediante la introducción de puentes disulfuro.

4.3. Los ensayos de síntesis de GOS apoyan la aplicabilidad de ambos mutantes en procesos industriales que requieren temperaturas elevadas.

5. Hemos llevado a cabo pruebas preliminares de inmovilización con la β-galactosidasa de *K. lactis* y con su mutante más termoestable, usando dos soportes magnéticos diferentes.

5.1. El soporte mPVA-POS resultó más adecuado que el soporte mPANI para inmovilizar ambas variantes enzimáticas. Esto podría explicarse por el tamaño más reducido de las partículas mPVA-POS, lo que implica un incremento en el área superficial específica de la partícula.

5.2. Los resultados sugieren un incremento en la capacidad para ser inmovilizado del mutante termoestable de la β-galactosidasa de *K. lactis*. Esto es corroborado por la significativa mejora de la eficiencia de inmovilización y de la capacidad de reutilización de la variante mutante, en comparación con la enzima silvestre en ambos soportes.

Appendix I I

Curriculum vitae

Publications

2017

Rico-Díaz, A., Álvarez-Cao, M. E., Escuder-Rodríguez, J. J., González-Siso, M. I., Cerdán, M. E., & Becerra, M. (2017) Rational mutagenesis by engineering disulphide bonds improves *Kluyveromyces lactis* beta-galactosidase for high-temperature industrial applications. *Scientific Reports. 7* : 45535.

Vizoso-Vázquez, A., Lamas-Maceiras, M., Fernández-Leiro, R., Rico-Díaz, A., Becerra, M., & Cerdán, M. E. (2017) Dual function of Ixr1 in trancriptional regulation and recognition of cisplatin-DNA adducts is caused by differential binding through its two HMG-boxes. *Biochimica et Biophysica Acta – Gene Regulatory Mechanisms. 1860*, 256-269 .

2014

Rico-Diaz, A., Vizoso Vazquez, A., Cerdán, M. E., Becerra, M., & Sanz-Aparicio, J. (2014) Crystallization and preliminary X-ray diffraction data of beta-galactosidase from *Aspergillus niger. Acta crystallographica.Section F, Structural biology communications. 70*, 1529-1531.

Conference communications

2014

Rico-Díaz, A., Cerdán, M. E., & Becerra, M. Evaluación de la producción de galactooligosacáridos en variantes de la beta galactosidasa de *Kluyveromyces lactis*. Poster communication to the XXXVII Spanish Society of Biochemistry and Molecular Biology Conference. (09-12 September 2014) (Granada, Spain).

Rico-Díaz, A., Vizoso-Vázquez, A., Cerdán, M. E., Becerra, M. & Sanz-Aparicio, J. Purificación y cristalización de la β-galactosidasa de *Aspergillus niger*. Poster communication to the XXIV Symposium of the Crystallography and Crystal Growth Specialized Group (GE3C), "Crystallography and Sustainability". (23-26 June 2014) (Bilbao, Spain).

2013

Rico-Díaz, A., Cerdán, M. E., & Becerra, M. Mejora de la termoestabilidad de la β-galactosidasa de *Kluyveromyces lactis* mediante mutagénesis dirigida. Poster communication to the XXXVI Spanish Society of Biochemistry and Molecular Biology Conference. (04-06 September 2013) (Madrid, Spain).

Escuder-Rodríguez, J. J., Fernández-Leiro, R., Rico-Díaz, A., González-Siso, M. I., & Becerra, M. Caracterización bioquímica de un mutante termoestable de la α-galactosidasa de *Saccharomyces cerevisiae*. Poster

communication to the XXXVI Spanish Society of Biochemistry and Molecular Biology Conference. (04-06 September 2013) (Madrid, Spain).

2012

Rico-Díaz, A., González-Siso, M. I., Cerdán, M. E., & Becerra, M. Improvement of thermal stability of *Kluyveromyces lactis* β-galactosidase. Poster communication to the 22nd Congress of the International Union of Biochemistry and Molecular Biology (IUBMB) and 37th of the Federation of European Biochemical Societies (FEBS). (04-09 September 2012) (Sevilla, Spain).

2011

Rico-Díaz, A., Vizoso-Vázquez, A., & Cerdán, M. E. Regulación del gen KlHEM13 por Kllxr1p en *Kluyveromyces lactis*. Poster communication to the XXXIV Spanish Society of Biochemistry and Molecular Biology Conference. (05-08 September 2011) (Córdoba, Spain).

Research Fellowships / Contracts

2015

Research stay fellowship (Inditex-UDC) Centre of Biological Engineering of University of Minho. (01 September 2015–30 November 2015).

2014

Travel fellowship to the XXIV Symposium of the Crystallography and Crystal Growth Specialized Group (GE3C), "Crystallography and Sustainability". (23-26 June 2014) (Bilbao, Spain).

2013

Predoctoral fellowship (Xunta de Galicia) Biochemistry area. Department of Cellular and Molecular Biology. University of A Coruña (15 April 2013 – 15 April 2016) (A Coruña, Spain).

2012

Travel fellowship to the 22nd Congress of the International Union of Biochemistry and Molecular Biology (IUBMB) and 37th of the Federation of European Biochemical Societies (FEBS). (04-09 September 2012) (Sevilla, Spain).

Predoctoral fellowship (University of A Coruña) Biochemistry area. Department of Cellular and Molecular Biology. University of A Coruña (May 2012 – 15 April 2013) (A Coruña, Spain).

2011

Travel fellowship to the XXXIV Spanish Society of Biochemistry and Molecular Biology Conference. (05-08 September 2011) (Córdoba, Spain).

Predoctoral contract (University of A Coruña) Biochemistry area. Department of Cellular and Molecular Biology. University of A Coruña (September 2011 – May 2012) (A Coruña, Spain).

2010

Master fellowship (University of A Coruña) (September 2010 – September 2011) (A Coruña, Spain).

2009

Undergraduate fellowship (University of A Coruña) Biochemistry area. Department of Cellular and Molecular Biology. University of A Coruña (September 2009 – september 2010) (A Coruña, Spain).

Participation in Research Grants / Projects

2012

Consolidación y estructuración de unidades de investigación competitivas del sistema Universitario de Galicia (Referencia: CN 2012/118).

Funded by Xunta de Galicia. Biochemistry area. Department of Cellular and Molecular Biology. University of A Coruña.

2012-2016

Principal Investigator: María Esperanza Cerdán Villanueva

2011

Bio-Enxeñeiria das Beta-Galactosidasas para uso na Industria Alimentaria (10TAL103006PR)

Funded by Xunta de Galicia. Biochemistry area. Department of Cellular and Molecular Biology. University of A Coruña.

2010-2013

Principal Investigator: María Esperanza Cerdán Villanueva

Research Stays

2016

Short stay of six days in the Physics-Chemistry Institute "Rocasolano" (IQFR) under the supervision of Dra. Juliana Sanz Aparicio (February 2016) (Madrid, Spain).

2015

Predoctoral stay during three months in the Centre of Biological Engineering (University of Minho) under the supervision of Dr. José António Teixeira (01 September 2015–30 November 2015).

Short stay of ten days in the Physics-Chemistry Institute "Rocasolano" (IQFR) under the supervision of Dra. Juliana Sanz Aparicio (March 2015) (Madrid, Spain).

2014

Short stay of three days in the Physics-Chemistry Institute "Rocasolano" (IQFR) under the supervision of Dra. Juliana Sanz Aparicio (October 2014) (Madrid, Spain).

2013

Short stay of ten days in the Physics-Chemistry Institute "Rocasolano" (IQFR) under the supervision of Dra. Juliana Sanz Aparicio (October 2013) (Madrid, Spain).

Other merits

"Chromatographic Techniques applied to the organic compound analysis" course organized by SAI of University of A Coruña (2016) (A Coruña, Spain).

"Basic and applied Research Metodology" course organized by University of A Coruña (2016) (A Coruña, Spain).

"ISI vs Scopus" course organized by University of A Coruña (2014) (A Coruña, Spain).

"Resources and documentary profits for the thesis and other academic works" course organized by University of A Coruña (2014) (A Coruña, Spain).

Official Master of Biotechnology certified by the University of A Coruña (2011-2012).

"Initiation to Biochemistry and Molecular Biology Research" course organized by the Spanish Society of Biochemistry and Molecular Biology Conference (SEBBM) (2010) (Córdoba, Spain).

4th Scientific conference "Celular therapy and regenerative medicine" organized by University of A Coruña (2010) (A Coruña, Spain).

"Clinic proteomic scientific meeting" organized by Biomedical Research Institute of A Coruña (INIBIC) (2009) (A Coruña, Spain).

Research interships in University Hospital Complex of A Coruña (CHUAC) (2009) (A Coruña, Spain).

3th Scientific conference "Celular therapy and regenerative medicine" organized by University of A Coruña (2009) (A Coruña, Spain).